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Immunity to herpesvirus infections of domestic animals

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Immunity to herpesvirus infections of domestic animals

A seminar in the CEC research programme on animal pathology, held in Brussels, Belgium, 4 and 5 December 1984

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C.E.C. SEMINAR ON IMMUNITY TO HERPESVIRUS INFECTIONS OF DOMESTIC ANIMALS

CONTENTS

PREFACE

Latent herpesvirus infections are of great importance in animal production and the phenomena of latency, reactivation and re-excretion of latent viruses impede their control.

Animals react to these infections by an immune response that can prevent not only the deleterious effects of an eventual reinfection but, sometimes, also, viral re-excretion and consequently dissemination.

Immunity to herpesviruses is therefore of primary importance and the comprehension of the underlying phenomena is the key to rational prophylaxis.

This book contains the reports given during a CEC Agricultural Research seminar held in Brussels on the 4th and 5th December, 1984. The seminar had as a central theme the immunology of herpesvirus infections. Sessions 1 to 4 are on the different features of the immune response to herpesvirus infections in general. Sessions 5 to 7 deal with some peculiar aspects of the immune response to specific herpesvirus infections of domestic animals. The last session gives a summary of the problems exposed during the seminar and forcasts further research.

We are greatly indebted to the Commission of the European Communities, for giving us the opportunity to organize this seminar in the Berlaymont building in Brussels.

We would also like to express our gratitude to all the people who helped us to make the seminar a success, to Prof. Y. BECKER of the Hebrew University of Israel for his help in drawing up the final programme and to all the staff of the Department of Virology-Immunology, Faculty of Veterinary Medicine, University of Liège, for their work in the preparation of this publication.

P.-P. PASTORET
E. THIRY
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July 1985.

SESSION 1

MOLECULAR ASPECTS OF HERPESVIRUSES

Chairman : J.H. SUBAK-SHARPE

Co-chairman : G. DARAI

Immunogenic components of herpesviruses

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Detailed information on immunogenic components of herpesviruses would certainly fill an additional symposium and most probably even discussing the components of only one animal herpesvirus will need so in the next future.

During herpesvirus infection a variety of virus specific proteins can give rise to an immune response. These proteins are presented in the right way one might even raise antibodies against the majority of the more than 50 herpesvirus-specific proteins. Therefore we would like to concentrate on the major immunogenic components and neglect all immune reactions dealing with cellular immunity, recognized by the humoral immune response.

Antibodies produced under natural infections or obtained from hyperimmunized animals, and nowadays monoclonal antibodies, are the tools to enravel, what we consider to be major immunogenic components. There is no question that antibodies from hyperimmunized animals recognize different antigen patterns than antibodies obtained from naturally infected convalescent animals.

Usually the major immunogenic components can already be detected by immunodiffusion, immunoelectrophoreses or immunoprecipitation assays. More sensitive techniques, like radioimmuno-assays and all kinds of blotting techniques, may clarify the picture. They might, however, also distract from the real in vivo situation. Certainly, the neutralizing capacity of an antibody helps to recognize a major immunogenic component.

Using recombinant DNA analysis it is possible to localize the major immunogenic components of a variety of herpesviruses by fine mapping on the genetic material. Most information exists on herpes simplex viruses and more and more is gathered recently about animal herpesviruses. Although all viruses have distinct features a high amount of colinearity in the genomes and gene functions has been observed (Roizman and Batterson, 1985; Davison and Wilkie, 1983). Obviously internal structures of herpesviruses like those of the nucleocapsid or specific enzymes, which are not under a strong immune pressure (as it is the case with the envelope and membrane-exposed components) seem to be more conserved. Only a few herpesviruses as known so far possess conserved sites on their envelope proteins. Major immunogenic

components are associated with membrane structures and are therefore recognized by the immune system early in infection. This makes sense since the organism has to eliminate infected cells and activate the defense mechanisms against such structures. There is general agreement that the major immunogenic components are comprised in glycoprotein structures. Profound knowledge exists already about the human herpesviruses types 1 and -2. Here, gB and gD make up major immunogenic components. Their essential role in neutralization and protection is out of question. They are also involved in fusion of cells (for review see Spear, 1985). Their expression on the cell surface during the infectious cycle has not fully been clarified. However, there is evidence, that a lot of the gD epitopes are recognized by neutralizeing antibodies. Using infected cells the anti-gD antibodies can much easier be absorbed out from convalescent sera than those antibodies directed against gB (Pauli and Ludwig, 1977). Batteries of monoclonal antibodies against these glycoprotein complexes are now in use to identify the epitopes of these membrane inserted proteins and determine their functional role (Pellet et al., 1985).

In the animal herpesvirus systems, knowledge about the major antigens is still premature. For bovine herpesvirus type 1 (BHV-1), glycoproteins in a molecular weight range of 75 K seem to be involved in neutralization. Monoclonal antibodies and the use of convalescent as well as monoprecipiting antibodies show that several proteins in molecular weight ranges between 50 K and 150 K are either in close steric neighbourhood on the virion or become closely associated during the detergent extraction, when the tertiary structure might be altered (Collins et al., 1984; Gregersen, 1983; Gregersen et al., 1985; Letchworth et al., personal communication; Van Drumen Little-Van den Hurk et al., 1984).

A similar situation is observed with the antigens and major immunogenic components of pseudorabies viruses. Here a 90-95 K glycoprotein gives rise to neutralizing antibodies and is regarded to be the major immunogenic component, although strong neutralization has also been observed with antibodies recognizing a 50 K glycoprotein (Bund, 1983; Hampl et al., 1984; Wathen and Wathen, 1984).

To simplify the picture one can argue that not all of the approx. 10 glycoproteins found in human or animal herpesviruses give rise to the same strong humoral immune response, but there are one or two (and often not more) single glycoproteins or glycoprotein complexes initiating the bulk of neutralizing antibodies. The fine structure of these complexes has still to be enravelled.

A unique situation extending the strong cross-reactivtiy of the major immunogenic components of HSV-1 and -2 has been elucidated with HSV, BHM virus and B virus (Ludwig, 1983), were undoubtedly several conserved antigenic determinants are present on gB, , when looked at all three viruses and on gD comparing HSV and B virus (Ludwig et al., 1983). Detailed studies on conserved structures in these two glycoproteins will soon show where evolutionary retained and where variable epitopes can be expected. Preliminary data indicate for example that on the glycoprotein "tree" of gB (HSV), modelled by Pellet et al. (1985) at least 10% of the epitopes are shared with those of the BHM virus glycoprotein. These are absent in the gD of these viruses and cannot be found in EB virus when the same panel of gB monoclonals to HSV gB was used for comparative studies (Ludwig et al., manuscript in preparation).

Modern tools, like monoclonal antibodies and the sequencing data of herpesvirus DNAs which enable to construct synthetic polypeptides, will soon allow deeper insights into conserved and variable structures of the major immunogenic components.

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SESSION_2

THE IMMUNE SYSTEM IN THE DEFENSE AGAINST HERPESVIRUSES

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IMMUNOBIOLOGY OF HERPESVIRUS INFECTIONS

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ABSTRACT

This review surveys some major immunobiological features of herpesvirus infections that must be considered in the design of immunological control measures. An overview is presented of the pathogenesis of various groups of herpesviruses. This is followed by a discussion of the mechanisms of defense which serve to impart resistance. Against initial infection, natural defense mechanisms predominate with natural killer cells, macrophages and interferons being the most important components involved. Adaptive immune responses are discussed with regard to their role in recovery from infection. Emphasis is directed at aspects of T lymphocyte mediated responses since such mechanisms appear to play dominant roles in immunity. Finally, the circumstances in which host responsiveness against herpesviruses can be immunopathological is briefly discussed.

INTRODUCTION

Herpesvirus are prevalent infectious agents in almost all species and with few exceptions have not been successfully controlled by vaccination schemes. Indeed, for many herpesviruses acceptable vaccines are unavailable and the agents are gaining in importance. Thus, there is some urgency to develop successful control measures either immunological or otherwise. The principal reason for our failure to develop successful immunologic control cannot be explained by the viruses' poor immunogenicity. Rather, it is because a state of latency is established after primary infection. In some instances, latent or chronic infection can involve components of the immune system itself and can lead to immune dysfunction. Despite intensive effort, we neither understand the molecular biology of latency nor know how to achieve its termination. The biological significance of latency is that it serves as a nidus of infection for reactivated disease in the infected host and as a source of virus to be spread to others. Thus immunological control measures must emphasize approaches to prevent initial infection and the establishment of latency or modulate immune responsiveness such that reactivated virus is inconsequential both as regards disease and dissemination to cohorts. In this article some immunobiological features of herpesvirus infections are discussed and the relevance of such observations to possible control measures are mentioned.

the treatment is by necessity cursory and selective and for more detailed discussion several recent reviews should be consulted (Shore and Nahmias, 1982; Kirchner, 1982, 1984; Rouse, 1984a, b, c; Lopez 1984a, b; Rouse and Horohov, 1984). Where possible references are made to appropriate reviews rather than to the original authors.

PATHOGENESIS OF HERPESVIRUS INFECTIONS

The members of the alphavirinae family of herpesviruses all cause acute infections which if not lethal are followed by clinical recovery with the virus remaining latent at some site in the nervous tissue. Of far more interest from an immunobiological perspective are viruses in the gammavirinae family which characteristically interact in a variety of ways with cells of the immune system. Examples include Epstein-Barr virus (EBV) of man and Marek's disease virus of chickens. Our comments will be confined to the former. EBV infection of young children is an unnoticeable event and entails an inapparent infection of B cells and cells in the oropharaynx (Epstein and Morgan, 1983). In contrast, primary infection of adolescents results in infectious mononucleosis in 50% of cases. The essential lesion is a polyclonal activation of B cells. The B lymphocytes undergo proliferation and activation which results in raised levels of antibody, including autoantibodies. One consequence is that immune complex disease can occur. Under normal circumstances, polyclonal B cell activation is terminated by an HLA restricted cytotoxic T cell and natural killer (NK) cell response. Ordinarily clinical and hematological recovery occurs, but there are exceptions. Most prominent among these are situations where individuals receive prolonged immunosuppressive therapy or suffer from an inherited X- linked immunoproliferative disorder (XLP) (Purtilo, 1980). In both instances, EBV infection can be fatal. With the XLP syndrome, which results from a primary T cell immunoregulatory defect, a variety of EBV induced diseases result from the uncontrolled B cell proliferation. These include space-occupying lesions, infarctions and hypergammaglobulinema. Occasionally monoclonal lymphomas occur. These seem to result from a cytogenetic error such as an 8:14 translocation similar to that which occurs in Burkitt's lymphoma (Manolov and Manolova, 1972).

Viruses of the beta herpesvirinae family may also establish a complex immunobiological relationship with their host. Best studied is the human cytomegalovirus (HCMV) (reviewed by Kirchner, 1984). Normally, HCMV is

not a cause of disease. However, if immunosuppression occurs through iatrogenic measures, other diseases or immune incompetence, then HCMV infection may lead to disease. Particularly prone to infection are the unborn and newborn, transplant recipients and those with secondary immunosuppressive diseases such as the acquired immunodeficiency syndrome. In such circumstances, HCMV may cause a variety of effects of which interstitial pneumonia, mononucleosis and hepatitis are major signs. Following HCMV infection, further immunosuppression occurs perhaps because of impairment of macrophage (MØ) function. The consequences of such immunosuppression is superinfection with a range of low grade pathogens of which Pneumocystis and Aspergillus are the usual culprits. Frequently the outcome is fatal.

RESISTANCE TO INITIAL INFECTION

Upon initial exposure to virus many natural defense mechanisms are available that can limit and curtail infection. The most comprehensive investigations on this topic have been with herpes simplex viruses (HSV) and the subject was lucidly reviewed by Lopez (1984a, b). Since the severity of initial herpetic infections is often a harbinger of recrudescent disease, natural immunity may have indirect effects on the latter and serve to limit the extent of latency. Adaptive immunity, which takes some time to develop, assumes more importance in facilitating recovery and enhancing resistance to reinfection (Rouse, 1984b).

A number of approaches have been used to identify natural defense mechanisms involved in resistance to herpetic infections. These include: (A) observations of naturally-occurring immune deficiencies to determine if enhanced susceptibility correlates with impaired activity of some defense mechanism; (B) measurement of effect on immunity of treatment to enhance or suppress some defense parameter; (C) observations of severity of infection in animal models of genetic resistance and finally (D) reconstitution experiments. None of these approaches prove ideal since rarely if ever is the activity of a single natural immune mechanism being measured. Thus, for example, the observation that herpetic infections are usually more severe when NK function is impaired as in the Wiscott-Aldrich syndrome of man (Lopez et al., 1983) and the beige gene mutation in mice (Shellam et al., 1981) has been taken as evidence for a crucial role of NK cells in resistance. However, since other cell types, such as polymorpho-

nuclear neutrophils (PMN) and MØ are also impaired in function, the conclusions remain equivocal. The observation that newborns are more susceptible to herpetic infections was explained by immature MØ function (Johnson, 1964) and, in fact, resistance could be increased by adoptive transfer of adult MØ (Hirsch et al., 1970). However, newborns have more than just an immaturity in MØ function and the adoptively transfered cells were not exclusively MØs.

Problems of a lack of selectivity also befoul the interpretation of experiments in which animals are treated so as to augment or suppress some natural defense mechanism for the purpose of determining the effect on subsequent infection. Many groups have used this approach to indicate a crucial role for MØ in natural resistance (reviewed by Morahan, 1984; Lopez, 1984a, b). Thus treatment with MØ poisons such as silica and carrageenan increased susceptibility. Unfortunately, the function of PMN, NK cells and perhaps other cell types involved in resistance are also affected. Observations that resistance is increased following the use of MØ immunopotentiators has also been used to indicate an essential role of MØ. Thus, C. parvum administration to mice increases their resistance to intraperitoneal infection with HSV, although interestingly not to the more physiological local routes of infection (McGeorge and Morahan, 1978). C. parvum activates MØ but also causes interferon induction which, in addition to its role as a MØ activator, has indirect antiviral effects and increases the activity of NK cells by a variety of mechanisms (Roy, 1984; Herberman et al., 1979). Consequently, the lack of selectivity provides a dilemma.

Two other results worthy of comment in which treatment with selective reagents was used to imply mechanisms of natural resistance against herpesvirus infections were those which showed that the in vivo administration of antiinterferon (anti-IF) (Gresser et al., 1976) and anti-asialo GM_1 serum (Bukowski et al., 1983) both increased susceptibility. The latter serum is claimed to affect only NK cell function, although it is well known that the asialo GM_1 marker is expressed on many other cell types including PMN and MØ (Herberman and Ortaldo, 1981). The observations with anti-IF serum strongly indicate a role for IF in resistance but since IF plays multiple roles in immunobiology, it is not possible from such experiments to conclude which aspect of immunity was being affected.

Some of the more persuasive arguments which indicate the mechanistic role of a natural defense component have come from studies of animal models of genetic resistance (reviewed by Lopez, 1984a, b). In such systems, in vitro and in vivo correlates of resistance can be evaluated in crosses and backcrosses of resistant and susceptible mice to determine whether functions in question segregate with resistance or independently. Another advantage is that these models use genetically homogeneous mice. Consequently, cell transfer experiments can be performed. Using the genetic model of resistance of HSV, Lopez (1984a) and Mogensen (1977), examined several mouse strains and showed that resistance was dominant and governed by two independently segregating, nonsex-linked, non H-2- linked loci. The pattern of resistance was true for all HSV strains investigated, whether the route of challenge was intraperitoneal, intravaginal or intraocular.

The results of several studies indicated that the host's hematopoietic defense system was responsible for genetic resistance and that resistance served to inhibit virus from reaching the central nervous system which appears to be the target organ in the mouse. The use of chimeras constructed from lethally irradiated susceptible mice reconstituted with bone marrow from resistant (F_1) mice was one line of evidence used. In addition, treatment of resistant mice with the bone seeking isotope strontium-89 abrogated genetic resistance as did prolonged treatment with oestradiol.

In analyzing the mechanisms involved in genetic resistance, most evidence pointed towards NK cells although some favor the MØ (Morahan, 1984) or the early interferon response (Engler et al., 1981) as the chief mechanism of resistance. Arguing principlely against MØ were experiments showing that MØ's ability to resist virus replication did not segregate with genetic resistance in F_1 and F_2 populations (Lopez, 1984b). The extent of NK cell activity correlated with the resistant state and NK cell responsiveness segregated with resistance in crosses and backcrosses. However, exceptions have been made noted by less enthusiastic advocates of NK cells (Kirchner, 1984). Nevertheless, the case for NK cells becomes even stronger when full realization of their heterogeneity is taken into account (Lopez, 1984b). The most likely alternative explanation is that the interferon response provides the direct defense mechanism. Both NK and interferons are now known to be highly heterogenous and interact with

each other intimately (Herberman and Ortaldo, 1981). Thus, interferons may serve to activate NK cells, recruit pre-NK cells, increase rate of NK cell recycling and render virus infected target cells more susceptible to NK cell lysis (Herberman et al., 1979). Certainly, in vitro examples can be found where the lytic function of NK cells is independent of interferon activity, but in the normal in vivo environment the two components serve parallel supportive roles to diminish the significance of herpetic infections.

In conclusion, natural resistance mechanisms constitute a significant barrier to herpesvirus infections. The proper functioning of these mechanisms is required by the host to survive their numerous encounters with the pathogens. With a thorough understanding of the effector cells involved and their interactions, it should be possible to develop ways of manipulating these to the advantage of the host. This topic has been discussed in detail elsewhere (Rouse and Lopez, 1984).

RECOVERY FROM INFECTION

There seems little doubt that whereas natural defense mechanisms play a crucial role in preventing or limiting initial infection, once infection has occurred resolution depends largely on the adaptive immune response (reviewed by Shore and Nahmias, 1982; Rouse, 1984a). Accordingly, if adaptive immunity is impaired, infections are more severe, often lethal, and are more likely to desseminate to critical organs such as the brain. Severe and lethal infections are commonly observed in the very young, the immunosuppressed such as renal transplant patients and in some models of primary T cell deficiency. In studies of both immunodeficient humans and immunosuppressed animals, the capacity to generate an antibody response can be normal in the face of a severe virus infection (Meyers et al., 1980; Lopez et al., 1974). In such individuals, cell mediated immunity (CMI) is often suppressed. Such observations, along with the knowledge that herpesviruses can disseminate in tissue culture in the presence of neutralizing antibody, are arguments commonly taken in support of a principal role for CMI in resolving infection. The fact that T lymphocytes seem designed to recognize virus infected cells (Zinkernagel and Doherty, 1979) (the principal target of recovery responses rather than free virus) and that the T cell response better correlates with lesion resolution than do antibody responses also supports a major role for T cell mediated

immune mechanisms. Nevertheless, the scenario is complex and it seems highly likely that the many mechanisms of T cell immunity interact positively and negatively with other aspects of immunity. The outcome with herpesvirus infections is usually rapid recovery, although examples of immunopathology have been noted (Rouse, 1984c) and, as is well known, immunity is never total since persistent or latent infection usually occurs. Since humoral immunity will be dealt with in the chapter by Dr. Norrild further mention will not be made of this aspect of adaptive immunity. Our discussion will emphasize T cell mediated mechanism and will be selective and incomplete because of space limitation. However, several more comprehensive reviews have been published recently (Shore and Nahmias, Rouse, 1984a, b; Kirchner, 1982, 1984; Rouse and Horohov, 1984).

Several approaches have been used to assess the role of adaptive immunity in herpetic infections, but perhaps the most analytical has involved observations of the course of infection in normal or compromised animals following passive transfer of antibody and adoptive transfer of defined T cell populations (reviewed by Rouse, 1984b). With such systems, evidence was obtained that several different mechanisms played a role in recovery (including antibody). Aspects of CMI appear as the principal mechanism of recovery, but whether delayed type hypersensitivity (DTH) or some other function of T cells such as cytotoxicity plays the dominant role remains debatable. In the mouse ear model, rapid elimination of infective virus from the ear of an acutely infected animal correlates with the appearance of DTH and this protection can be transferred to infected recipients with Lyt1+2- T cells, the only T cell subset known to mediate DTH against HSV in the mouse (Nash and Gell, 1981). However, by means of adoptive transfers of immune lymphocytes, others have shown that Lyt1-2+ cytotoxic T lymphocytes (CTL) populations can also clear virus (Larsen et al., 1984). Moreover, it is known that animals rendered tolerant for DTH still recover from infection. Such observations indicate that several T cell systems serve similar overlapping functions of clearing virus from tissues. Removal of replicating virus from epidermal or mucocutaneous surfaces is usually achieved without tissue damage (reviewed by Rouse, 1984c). However, there are instances where prolonged responses result in immunopathological reactions. Some of these are discussed subsequently.

IMMUNOBIOLOGICAL ASPECTS OF ANTI-HERPES T CELL IMMUNITY

Herpesvirus specific T cell responsiveness can be measured in a variety of ways. The most usual, but analytically least helpful, has been to measure antigen induced proliferation in peripheral blood leukocyte or lymphoid cell suspensions. Several functional cell types of T cells can be induced to proliferate and under some circumstances herpesvirus antigens may even act as B cell mitogens (Kirchner, 1982). Moreover, proliferation is dependent on the presence of appropriate antigen presenting cells and the elaboration of proliferation factors such as interleukin 2 (IL-2) (Rouse, 1984a). The response of cell mixtures is also subject to inhibition and regulation by antigen specific suppressor cells. Consequently, unless efforts are made to separate lymphocyte subsets prior to stimulation it is difficult to relate levels of lymphocyte proliferation to any functional parameter of T cell immunity. A discussion of how lymphoproliferation correlates with viral pathogenesis and in particular with the onset of recrudescent herpetic disease was published elsewhere (Horohov et al., 1984).

Several in vitro assays of T cell reactivity to herpetic antigens correlate more closely with probable in vivo function. These include assays for CTL and their precursors (CTL-P), assays for helper lymphocytes (HTL) and their precursors (HTL-P) and finally assays of suppressor lymphocyte (STL) activity. It is clear from work done in several systems, including those involving herpesvirus antigens (Rouse, 1984a), that these activities are usually expressed by different lymphocyte subsets and that the series of events which lead to their activation are quite different. Thus, all 3 functional lymphocyte subsets precursors must be activated by viral antigen in the membrane of an antigen presenting cell (APC), usually a MØ. It is becoming increasingly evident that different APC serve to activate the various subsets of lymphocytes (Unanue, 1984). Thus, HTL-P are activated by IA positive APC, CTL-P by IA negative (as well as perhaps positive) APC and STL by IJ positive APC. Presumably, subtypes of APC are differentially represented at various locations in the body. This could well provide an explanation for the observation that different routes of infection may affect the type of T cell immune response that occurs (discussed subsequently).

The delayed type hypersensitivity (DTH) response is the paradigm of an in vivo T cell mediated CMI response. This response has been carefully investigated in regard to HSV in the mouse and has been reviewed (Nash and

Gell, 1984). Particular attention was paid to determining the cell types involved in the response, its regulation, the antigens and routes of immunization required for DTH induction and the role of DTH as a protective or immunopathological reaction. Anti-herpesvirus DTH was established following local infection, but not after exposure via the intravenous route. In this latter circumstance, suppressor cells were induced which served to inhibit DTH at both afferent and efferent levels (Schrier et al., 1983). It is instructive to emphasize some observations made with viruses other than herpes since similar effects could also occur with herpesviruses in nonmurine systems. In influenza, for example, following infection with replicating virus, two subsets of DTH mediating T cells were induced - one restricted by class I and the other by class II (IA) major histocompatible antigens (Ada et al., 1981). After exposure to non-replicating virus, only the IA restricted cells were induced. Moreover, such cells were involved in immunopathological reactions rather than protective immunity. Upon exposure to live virus, the induced response was protective. This occurred because the class I mediated DTH cell provided protection and in addition, immunopathological reactions were prevented by a concomitant induction of suppressor cells (Liew and Russell, 1983). Clearly the nature of antigens used in vaccines is important since certain forms of non-infective antigen preparations could set the stage for immunopathology when subsequently exposed to replicating virus.

IMMUNOPATHOLOGICAL REACTIONS INVOLVING HERPESVIRUS INFECTIONS

In the majority of circumstances the outcome of an adaptive immune response to herpesvirus infection is protection. However, sometimes significant tissue damage occurs. Such immunopathological reactions vary widely in mechanism and include chronic T cell mediated responses to persistent antigen, toxic immune complexes and perhaps reactions mediated by IgE. The general topic of the role of immunopathological reactions in herpesvirus infections was reviewed recently (Rouse, 1984c).

There are several sporadic examples of immunopathology such as immune complex lesions in skin and the glomerulus after infection under certain circumstances. Perhaps the best candidate syndrome involving herpesviruses in which the mechanism of pathogenesis is essentially immunopathological is that of disciform keratitis. This inflammatory reaction of the corneal stroma occurs following HSV recrudescence in the eye (Kaufman,

1978). Frequently the reaction can become more marked with each recrudescence and corneal blindness may result. In fact HSV infection is a common cause of corneal blindness in the USA. Epithelial keratitis results directly from virus replication in epithelial cells and responds very well to treatment with antivirals. Indeed it was in the treatment of epithelial keratitis that antiviral drug treatment was first found to be clinically useful (Kaufman et al., 1962). However, keratitis affecting the stroma responds less well to antiviral drugs and needs judicious treatment with corticosteroids for successful management. It is usually not possible to demonstrate infectious virus in the stroma, but from work done on clinical material and in animal models, viral antigens can often be detected in keratocytes which are surrounded by inflammatory lymphocytes. (Metcalf and Reichert, 1979).

The mechanism of herpetic keratitis has been studied in both rabbits and mouse models. It remains unclear as to the exact immunopathological sequence of events that causes the reaction but evidence has been marshalled to support a role for immune complex lesions as well as a chronic T cell response mediated by more than one subset of T cells (Russell et al., 1984). It is important to be aware of the possibility of inducing or sensitizing for subsequent immunopathological reactions in designing future vaccines for use against herpesviruses.

CONCLUSIONS AND SPECULATIONS

In the development of new vaccination approaches for use against herpesviruses some immunobiological aspects should be taken into account. Of principal relevance are the influences that route of vaccination and nature of vaccine can have on the type of immune response induced. For example, whereas infection via the subcutaneous or intramuscular routes induces a positive response, oral, intravenous (i/v), or ocular infection favors the generation of suppressor cell responses (Rubin et al., 1984; Nash and Gell, 1981; Whittum et al., 1984). The target cells for such suppression may vary. With i/v infection, APC as well as lymphocyte precursors and effectors that mediate DTH act as the targets (Schrier et al., 1983). Conditions have also been established in which suppressor cells are induced which regulate the activity of herpesvirus specific CTL (Horohov et al., 1984b). The reasons why a given route of infection preferentially induces suppression needs to be established. However, the

most likely explanation is that APC involved in induction of the different types of responses are differentially represented in each location.

It is clearly evident that the nature of antigen can markedly affect the type of immune response induced. However, we know little about the nature of herpesvirus antigens involved in T cell induction or target cell recognition. For CTL induction and recognition against HSV they seem to be glycoproteins (Rouse, 1984a). In contrast against CMV, nonstructural early proteins appear to be the antigens involved (Reddehase et al., 1984). It could be that determinant groups involved in CTL and HTL induction differ from those involved in suppression. This is the case with less complex antigens such as lysozymes (Adorini et al., 1979). Hopefully, this topic will become clarified once more becomes known about the immune response to herpesvirus-specific synthetic peptides.

With herpesviruses, our experience has been that non-replicating viruses and isolated viral glycoproteins represent very weak antigens in regards to the in vivo induction of CTL and helper T cells involved in CMI responses (Rouse, 1984a). For instance, we have developed highly sensitive limiting dilution assays to quantitate numbers of CTL, CTL-P and IL-2 producing HTL precursors in lymphoid tissues of mice following exposure to HSV (Rouse and Wagner, 1984; Prymowicz et al., 1984). Our estimates of numbers of both CTL-P and HTL-P fall in the range of 1/3000 - 1/8000 after recent exposure to infectious virus and 1/15000 in long term memory populations. Interestingly, exposure to nonreplicating virus and glycoprotein antigens may fail to induce any IL-2 producing HTL-P, although helper T cells involved in antibody production are elicited. In contrast, CTL-P are induced although the memory CTL-P are markedly reduced. Our results demonstrate the ineffectiveness of inactivated vaccines at stimulating appropriate anti-herpes virus immunity and also indicate that to induce a broadly reactive balanced T cell response, replicating vaccines are required. However, it is likely that with more careful analysis of antigen presentation requirements and the use of adjuvants, isolated viral proteins and peptides will prove to be suitable immunogens against herpesviruses. There could also be a place for anti-idiotype vaccines (Rouse, 1984c).

It is important to continue research in the field of immunoregulation with with respect to viral infections. The clinical application of our

knowledge is that eventually it may prove feasible to tailor the type of immune response obtained. For example, if recovery is found to correlate best with the HTL response, we need to use immunogens and routes of vaccination which best stimulate this type of response. In cases of herpesvirus immunopathology, such as corneal stromal keratitis, ways must be found to induce regulatory suppressor cells. In situations of recrudescence, which some have suggested results from the overactivity of suppressor T cells (Sheridan et al., 1982), we need to find modes of immunization that will inhibit such suppression and so limit the severity of recrudescence. Recently, basic studies on the nature of suppression have indicated that some forms may be overcome by the administration of additional IL-2 (Green et al., 1983). In our own studies on HSV specific CTL suppression, we have also observed such effects, and have also shown that the in vivo supplementation of IL-2 may markedly facilitate recovery from infection (Rouse et al., 1984b). Such studies lead us to anticipate that IL-2 administration could minimize the severity of recrudescent disease, a topic currently under further investigation in our laboratory.

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THE BIOLOGY OF TWO HERPESVIRUS INFECTIONS OF DOMESTIC ANIMALS.

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ABSTRACT.

One of the major features of herpesvirus infections of animals is latency. From time to time detectable episodes of virus shedding may occur, both apparently spontaneously and as a result of certain endogenous and exogenous stimuli. A number of experimental systems have been investigated to study the phenomenon of latency and recrudescence, mainly using herpes simplex virus infection in the mouse model. However, there are a number of other animal species that can be studied where the herpesvirus is in its own natural host-virus system. Two such examples, the cat and pigeon herpesviruses, are described here. Both of these produce a latent infection in their hosts, and can be reactivated under well-defined conditions either as a result of natural, physiological stresses, or from more artificial stimuli. As a result of these observations, some conclusions are drawn about the biological role of latency.

INTRODUCTION.

One of the major features of herpesvirus infections of animals is the phenomenon of latency. Latency may be defined as the masked persistence of virus in the host, so that it cannot be detected by conventional virological techniques. From time to time, however, detectable episodes of virus shedding (re-excretion) may occur, both apparently spontaneously and as a result of certain endogenous or exogenous stimuli, and which may or may not be accompanied by clinical signs (recrudescence). Thus in people, herpes simplex virus (HSV) recrudescence tends to occur after physiological stresses such as menstruation or pyrexia (Roizman, 1965), and in animal herpesvirus infections stresses such as a change of housing or the reproductive period may induce virus re-excretion (Gaskell and Povey, 1977, 1982; McFerran et al., 1984; Vindevogel et al., 1985). Re-excretion may also be stimulated in many species by means of corticosteroid or other immuno-suppressive treatment (Sheffy and Davies, 1972; Gaskell and Povey, 1973; Vindevogel et al., 1980b; Wittmannn et al., 1982), though the exact mechanism of this is unknown.

A number of experimental systems have been investigated in order to study the phenomenon of latency and recrudescence. The majority have concentrated on human HSV infection in the mouse model, the mouse being chosen not only because of convenience and our immunological knowledge of the host, but also because of its low background level of spontaneous reactivation compared to some other laboratory species (Sekizawa et al., 1980; Openshaw, 1984). A variety of methods have been used in

the mouse to induce re-excretion, including corticosteroid treatment (Underwood and Weed, 1974), cyclophosphamide (Kurata et al., 1978), skin trauma (Hill et al., 1978) and ultraviolet light (Blyth et al., 1976). Possible mechanisms for the establishment, maintenance and control of HSV latency, particularly with respect to experimenatal findings on the mouse model, have been discussed by Blyth and Hill (1984).

However, there are a number of other animal species that may be studied where the herpesvirus is in its own natural host-virus system. Studying latency in the proper animal species reduces errors that may arise as a result of experimental contrivance, for the same herpesvirus infection may give different results both within and between species. In veterinary medicine compared to human medicine however, virus re-excretion or shedding ("reactivation" in the mouse model) is considered more important than recrudescent disease, for it is the epidemiological implications of the re-excreting animal in a herd or group that are important, rather than recrudescent disease in the individual.

This paper describes two herpesvirus infections of animals; Felid herpesvirus 1 (FHV 1) and Pigeon herpesvirus 1 (PHV 1). Both of these produce a latent infection in their hosts, and can be reactivated under well-defined conditions either as a result of natural, physiological stresses, or from more artificial stimuli. As a result of these observations, some conclusions are drawn about the biological role of latency.

FELID HERPESVIRUS 1 (FHV 1).

FHV 1 is an alphaherpesvirus of cats. It is a respiratory pathogen producing a disease known as feline viral rhinotracheitis. In contrast to the broader host range of some other herpesviruses such as ADV or HSV, the virus only affects domestic cats and other Felidae (Povey, 1979). All isolates so far examined from many parts of the world show antigenic homogeneity on the basis of conventional serological cross-neutralisation tests (Crandell et al., 1960; Bittle et al., 1960; Johnson and Thomas, 1966), although more refined serological techniques have not been used. Recent work using restriction enzyme analysis of the viral DNA has confirmed this high degree of similarity between strains which in general is reflected in the relatively uniform biological behaviour of isolates (Hermann et al., 1984). However strains of modified virulence do exist, having been produced in recent years for vaccines (Slater and York, 1976; Davies and Beckenhauer, 1976; Bittle and Rubic, 1975).

No serological relationship has been demonstrated between FHV 1 and a second feline herpesvirus associated with urolithiasis (Fabricant and Gillespie, 1971), nor with several other alphaherpesvirus of other species (Povey, 1979).

FHV 1 is highly infectious to susceptible cats, infection occurring via the intranasal, oral or conjunctival routes. In the acute phase of the disease, the major sites of virus replication are in the nasal septum, turbinates, nasopharynx and tonsils; conjunctivae, mandibular lymph nodes and trachea are also often involved (Crandell et al., 1961; Gaskell and

Povey, 1979a). A viraemia has only rarely been reported. A possible genital tropism has been investigated experimentally, but is not thought to be of significance under natural conditions (Bittle and Peckham, 1971; Hoover and Griesemer, 1971).

The virus is a comparatively labile virus, surviving under typical external environment conditions for less than a day (Povey and Johnson, 1970) and susceptible to all common disinfectants (Scott, 1980). As an aerosol, it is relatively unstable at midrange and higher relative humidities (Donaldson and Ferris, 1976).

Incidence and clinical signs.

FHV 1 is a highly successful virus in cats. It is widespread throughout the world (Crandell, 1973) and together with feline calicivirus, accounts for the majority of cases of feline respiratory disease (Kahn and Hoover, 1976; Gaskell and Wardley, 1978). Clinically it is the most significant of the feline respiratory pathogens. Serological surveys prior to vaccination demonstrated FHV 1 serum neutralising antibody titres in 26-70% of cats, depending on the nature of the sample population (Studdert and Martin, 1970; Povey and Johnson, 1971; Ellis, 1981). In general infection is less common in isolated household pets than in colony animals. However, the household pet is artificially separated from other cats by man; an open colony situation being a truer reflection of how virus and host evolved together. Once the virus has gained access to a susceptible colony, often by means of a clinically normal carrier, the disease rapidly becomes endemic, its presence being noted by the existence of chronically affected animals with recurrent or persistent signs. Outbreaks of acute disease may also occur, particularly in young kittens when they lose their passive immunity.

Acute infection with FHV 1 is characterised by depression, sneezing and ocular and nasal discharges (Crandell et al., 1961; Gaskell and Povey, 1979a). There is usually a fever and loss of appetite. Conjunctivitis, hypersalivation, and sometimes dyspnoea and coughing may develop and there may be a recurrence of the pyrexia. A leucocytosis with a left shift is present throughout the course of the disease. Other, rarer manifestations have been reviewed by Gaskell and Wardley (1978) and Povey (1979). Mortality may be high in young or debilitated cats and virus generalisation may occasionally occur. The majority of clinical signs have usually resolved in 10-20 days, but some animals may be left with chronic sequelae such as conjunctivitis, rhinitis, or sinusitis. These are probably mainly due to severe mucosal damage and secondary bacterial infection, but may also occur as a result of viral recrudescence.

Experimental infection and induction of re-excretion.

The most common route of infection used experimentally is intranasal, although several other routes have been investigated (Povey, 1979). The incubation period is 2-7 days, though it may be longer and has been shown to be dose-related (Gaskell and Povey, 1979a). Virus is shed in oropharyngeal, nasal and conjunctival secretions for a period of one to three

weeks, titres of up to $10^{4.8}$ (mean 10^{4}) $TCID_{50}$ per ml. of secretion being reached (Gaskell, 1975).

Experimentally it has been shown that the majority of FHV 1 recovered animals are latently infected virus carriers (Gaskell and Povey, 1973, 1977). Re-excretion may occur spontaneously, but is most likely to occur following stress. Under experimental conditions it has been shown that a change of housing may induce virus shedding in 18% of 22 FHV 1 recovered cats on 15% occasions, and corticosteroid in 69% of 32 cats on 54% occasions (Gaskell and Povey, 1973, 1977). Climatic stress appeared to be ineffective in inducing re-excretion. The apparently spontaneous shedding rate was 0.9% on any one day. In these studies, a total of 82% of FHV 1 recovered cats shed endogenous virus on at least one occasion and 45% shed virus spontaneously or under "natural" stress conditions and thus could be considered epidemiologically important. Similar findings have been reported by Ellis (1981) and Goddard (1984).

A lag period occurred before onset of virus shedding of from 4-11 days (mean 7.2) after corticosteroid treatment, and from 4-10 days (mean 7.2) after re-housing (Gaskell and Povey, 1973, 1977). The duration of virus shedding ranged from 1-13 days (mean 6.5) after corticosteroid and 4-7 days (mean 7.2) after re-housing. Titres of virus shed were generally lower (p less than 0.01) than in the acute stage of the disease, although amounts of up to $10^{5.8}$ (mean $10^{3.1}$) $TCID_{50}$ per ml. of secretion were recorded (Gaskell, 1975). In some cases (72% following corticosteroid stress, 30% following re-housing) shedding was accompanied by recrudescence of mild clinical signs, though occasionally signs were seen in carriers unassociated with detectable episodes of re-excretion.

It appeared that there was a refractory period following an episode of corticosteroid induced re-excretion during which further administration of corticosteroid was less effective (Gaskell and Povey, 1977). Cats which did re-excrete as a result of treatment had last shed virus on an average of 16 weeks before, whereas cats which did not re-excrete had experienced their last episode of virus shedding on an average of only eight weeks before (p less than 0.01). Considerable variation was apparent however, both within and between individuals.

The site or sites of latency have not been completely elucidated for FHV 1. Despite several unsuccessful attempts in the past to isolate virus from the trigeminal ganglia and a number of other tissues of latently infected cats (Plummer et al., 1973; Gaskell and Povey, 1979b; Ellis, 1982), recently virus has been isolated using a tissue fragment culture technique from the trigeminal ganglia of a small proportion, 3 (18%) of 17 recovered animals (Gaskell and Goddard, 1984; Gaskell et al., 1985). However, it remains to be seen if this is the major or only site of viral persistence in this species.

Natural transmission of the disease.

The major method of spread of FHV 1 is by direct cat-to-cat contact, either from animals in the acute stage of the disease, or from shedding carriers. There is no evidence of vertical transmission. Indirect transmission may also occur,

but probably less frequently, and only within the close confines of a cattery. Carrier cats are of considerable epidemiological significance: they are widespread in the population, and in an endemic cattery, constitute the majority of recovered animals. In the field situation, surveys of clinical normal cats have shown an apparently spontaneous shedding rate of 1-2% (Wardley et al., 1974; Ellis, 1981), and Gaskell (1975) recorded FHV 1 re-excretion in 3 of 75 cats 9-12 days after entering a boarding cattery. It seems probable, therefore, extrapolating from the experimental studies, that other stresses, such as going to stud, to a cat show, or entering a new cat colony, may also induce similar episodes of re-excretion.

Although carriers are undoubtedly a source of infectious virus and can initiate outbreaks of disease, it seems that cross-infection is less easily achieved from a shedding carrier than from an acutely infected animal (Gaskell and Povey, 1982). Thus under experimental conditions, fairly intimate contact of several days' duration is necessary before successful transmission may occur. This is presumably because discharges are usually more copious and in slightly higher titre in acutely infected animals than in carriers.

Under more natural conditions, however, it is likely that the greatest importance of the carrier lies in its ability to transmit the virus within the close contact of family groups, thus enabling it to perpetuate the host-virus relationship into the next generation. In studies on the possible transmission of FHV 1 from carrier queens to their kittens, the shedding rate from queens in the 10 week post-partum period did appear to be marginally increased above the spontaneous rate: four of ten queens re-excreted virus and four kittens from three litters developed a contact infection (Gaskell and Povey, 1982). None showed clinical signs and were presumably infected under cover of passive immunity. Two shed virus for one day only and did not become carriers, and two shed virus for two to three weeks and were subsequently shown to have become carriers: their SN antibody titres rising from less than 1 in 4 and 1 in 8 prior to re-excretion, to 1 in 96 and 1 in 128 after. The establishment of a latent carrier state under cover of passive immunity in animals which then became sero-negative, has also been shown for HSV infection in mice (Sekizawa et al., 1980).

From other studies it appears that there are many cases where carrier queens shed virus either when their own or other kittens in close contact are unprotected by maternal antibody, and cases of acute disease result (Povey and Johnson, 1967; Crandell, 1971). Although such cases will aid viral dissemination in the short term, in many ways it is not a good method for the virus to perpetuate itself in its host as mortality and also chronic sequelae in young kittens can be high. The findings outlined above, however, show that on some occasions at least, the cat has an ideal method of perpetuating a balanced virus-host relationship which does not depend on the development of clinical disease. This then leads to the establishment in the next generation of the latent carrier state, so the cycle is complete.

Immune control of re-excretion.

Immunity to re-infection following primary infection with FHV 1 is not very complete or persistent; an animal may be re-infected within six months of a primary infection, although such cats show only mild clinical signs and a reduced period of virus shedding (Walton and Gillespie, 1970). A number of vaccines have been developed for use in controlling the disease, including live attenuated vaccines given intranasally or systemically, and inactivated vaccines given systemically. These have been reviewed elsewhere (Gaskell, 1981). All vaccines protect to a large extent against clinical signs following challenge, though the intranasal route may be marginally more effective. However it has been shown that following systemic vaccination, cats generally replicate virus for several days after challenge and may subsequently become latent field virus carriers (Orr et al., 1978). Following intranasal vaccination, however, with a cold-adapted strain of FHV 1, virus replication after challenge appeared to be minimal, and in the short term at least, no animals appeared to have become carriers (Orr et al., 1980; Cocker et al., 1984). Thus some vaccinated animals, whilst themselves protected from clinical disease, may still be a source to others of infectious field virus.

Whether or not repeated vaccination helps control viral re-excretion episodes is not known. There appears to be a natural refractory period after an episode of re-excretion (see earlier) and so it seems possible that externally administered antigen may also help control re-excretion.

PIGEON HERPESVIRUS 1 (PHV 1).

PHV 1 is predominantly a respiratory pathogen of pigeons, although it also occurs in budgerigars and is antigenically indistinguishable from falcon or owl herpesviruses (Mare and Graham, 1973; Purchase et al., 1972). However it is probable that pigeons are the predominant natural host of PHV 1, and that infection in budgerigars, and possibly other species, is an epidemiological dead-end. Thus, although a spectrum of clinical signs may be seen in infected pigeons, in budgerigars, PHV 1 provokes, both naturally and experimentally, a fatal hepatitis (Vindevogel and Duchatel, 1977; Vindevogel et al., 1978, 1980c).

A similar situation exists with Aujeszky's disease virus (ADV) (Suid herpesvirus 1) where pigs are the main host, and infection in other species, such as cattle, dogs and cats, is of no epidemiological significance (Aguilar-Setién et al., 1979a, 1979b).

Incidence and clinical signs.

PHV 1 infection is widespread in domestic pigeon populations. In Belgium, specific antibodies have been detected in the sera of 84% of clinically normal pigeons and 63% of pigeons affected with acute respiratory illness. PHV 1 has been isolated in 60% of dove-cots permanently affected with respiratory disease and from 82% of pigeons affected with acute

respiratory troubles (Vindevogel et al., 1981; Vindevogel and Duchatel, 1978). A similar situation has been described in Germany and France (Heffels et al., 1981; Landre et al., 1982). Virus has also been isolated from pigeons coming from dove-cots where all birds are devoid of detectable specific antibodies (Landre et al., 1982).

Under natural conditions, clinical disease is predominantly seen in primary infection of young pigeons derived from parents free of the infection, or in carriers of the virus with the help of debilitating factors (Vindevogel et al., 1980a, 1981). Experimental disease may be reliably produced in squabs born from virus-free parents (Vindevogel and Pastoret, 1981).

Classical signs of PHV 1 infection in pigeons are conjunctivitis, rhinitis, and focal necrosis, in the mouth, pharynx and larynx. In some cases, particularly in pigeons weakened by debilitating factors such as parasitic disorders or secondary bacterial invaders, viral dissemination may also occur and lesions may be observed in liver, spleen, kidney and pancreas (Cornwell and Wright, 1970; Boyle and Binnington, 1973; Vindevogel et al., 1975; Vindevogel and Pastoret, 1981).

Experimental infection and induction of re-excretion.

After inoculation with PHV 1 by pharyngeal painting, susceptible squabs excrete virus for a minimum of 7 to 10 days (Vindevogel et al., 1980b). The typical lesions appear 1 to 3 days after infection when the viral excretion reaches its highest titre. Virus usually remains confined near the site of inoculation, although viraemia may occur.

Mild episodes of recurrence, without clinical signs, occur spontaneously (Vindevogel et al., 1980b). High titres of specific antibodies do not prevent these recurrences, and conversely, recurrent episodes do not occur more frequently when the animals are nearly devoid of specific antibodies.

PHV 1 re-excretion can be provoked experimentally by cyclophosphamide (Cy) treatment, and this period of re-excretion may be accompanied by lesions of varying severity and some birds may die (Vindevogel et al., 1980b; Vindevogel and Pastoret, 1981). In one group, all birds re-excreted infectious virus starting 2 to 5 days after the first injection of Cy for between 1 to 10 days: amounts of virus shed were similar to those seen in the acute phase of the disease. The mechanism by which Cy initiates viral re-excretion is not known. It may be due to a direct cytotoxic effect on latently infected cells or indirectly through the effects of Cy on B and T lymphocytes (Coignoul and Vindevogel, 1980). This effect of Cy on the immune system may account for the more severe clinical syndrome and higher mortality seen in Cy treated birds (Vindevogel et al., 1980b; Vindevogel and Pastoret, 1981).

Natural transmission of the disease.

As far as is known, egg transmission of the disease does not occur. No genital form of the disease has been detected and neither virus nor its antigen could be demonstratred in cell cultures derived from embryos from infected parents (Vindevogel and Pastoret, 1980, 1981).

Although virus may be spread horizontally from acutely

infected birds, infection is mainly perpetuated by means of carriers. Thus in a flock of pigeons infected with PHV 1, virtually all the mature birds are asymptomatic carriers of the virus and are thus a potential source of infection to their offspring, and to any susceptible in-coming birds. Apparently spontaneous episodes of re-excretion may occur, and since significant amounts of virus are shed, such birds presumably may transmit the infection (Vindevogel et al., 1980b). However there is some evidence that these spontaneous episodes are not as long-lasting as Cy-induced or natural stress-induced episodes of re-excretion, and thus they may not be of such epidemiological significance.

Studies have shown however, that virtually all pigeons will experience an asymptomatic re-excretion episode of reasonable duration during the reproductive period. Most of them re-excrete virus soon after the hatching period, when they regurgitate "milk" for their squabs (Vindevogel et al., 1985). After weaning (12th week after hatching) all parents cease shedding virus. The squabs become infected but are protected from the disease by passive immunity of parental origin, conferred to the squabs through the egg yolk. Most of the squabs themselves then become asymptomatic carriers after this initial infection, though they are very soon devoid of detectable antibodies. However, infection may be unmasked by Cy treatment, and presumably such squabs may themselves be capable subsequently of transmitting the virus to the next generation.

Under natural conditions therefore, there is a sophisticated equilibrium between the virus and its host that prevents the occurrence of disease. Nearly all the birds are infected and the infection can be perpetuated with a small number of infected birds, without exogenous introduction of wild virus.

Immune control of re-excretion.

Primary infection of pigeons with a pathogenic strain of PHV 1 (Vindevogel et al., 1982) prevents recurrence of the disease after re-infection. The protection is sufficient to inhibit viral multiplication in some birds. Both attenuated and oil-adjuvanted inactivated vaccines have been developed and are effective in reducing primary viral excretion and symptoms after challenge (Vindevogel et al., 1982a and b). Nevertheless neither vaccine was able to prevent the appearance of carriers since most of the pigeons subsequently re-excreted virus after Cy treatment. However vaccination helps to prevent spontaneous viral re-excretion and therefore helps to control viral dissemination. If animals are vaccinated with an inactivated vaccine after challenge with a virulent strain, experimental re-excretion is also reduced.

Nevertheless, vaccination is not the complete answer for the control of PHV infection, since young pigeons are often infected shortly after hatching and vaccination does not prevent the development of a carrier state or subsequent episodes of re-excretion.

DISCUSSION.

These animal-herpesvirus systems demonstrate the phenomenon of latency in the natural host. In each system, well-defined re-excretion episodes may be provoked by means of artificial stimuli such as corticosteroid or cyclophosphamide. In addition, re-excretion episodes may also be stimulated by means of natural stresses such as reproduction or a change of housing.

There are several advantages to using natural host-virus systems to study the phenomenon of herpesvirus latency. Firstly, since virus and host probably evolved together over a considerable period, the biological and epidemiological significance of spontaneous and natural shedding episodes may be assessed. In addition, observations on the mechanism of establishing, maintaining, reactivating, and controlling latency are probably more valid since they also relate to a naturally evolved relationship. In contrast, laboratory models such as the mouse-HSV system are easier and cheaper to maintain and are better defined genetically and immunologically.

To be a successful parasite, a virus needs to be able to ensure its transmission to as many animals as possible in each generation. Herpesviruses are in general relatively fragile outside their host, and therefore they cannot rely on external survival for their long-term persistence. In general, they have no reservoir or alternative hosts. Transmission by direct contact spread from acutely infected to susceptible animals certainly occurs, but requires a sufficient number of susceptible animals in the population and sufficient opportunities for contact between them or the virus will die out. Herpesviruses have there evolved the highly successful method of perpetuating themselves in a population by means of latently-infected carriers. Such carriers are only of biological significance, however, if shedding episodes coincide with the presence of susceptible individuals in close enough contact for transmission to occur.

In a population where the disease is endemic, the time when this is most likely to occur is just before or during weaning when the parent and young are still in very close contact but the young have become susceptible to the virus due to the waning of passive antibody.

Studies on the cat and pigeons have shown however that the timing of the shedding episode during this post-partum period is crucial. If it is too soon, only transient infection will occur and the offspring might not themselves become carriers. If it is too late, i.e. when maternal antibody has completely waned, then the clinical disease may be too severe and the host's survival compromised. Ideally the shedding episode should occur whilst the offspring are still protected from clinical disease, but not from virus shedding and the subsequent development of a latent carrier state. In the pigeon particularly, but to a lesser extent also in the cat, such a balanced virus-host relationship does occur, ensuring survival of the virus in as many animals as possible in the next generation, so that the cycle is complete. Indeed it may be that even in the cat, under more natural conditions, shedding

may occur in more animals during the post-partum period than was apparent from these studies: the cats used in this work had previously been induced to shed artificially on a number of occasions, and this may have induced a relative "refractory period", making them less likely to shed after parturition.

In a herd or family group, where the young are of similar age and are in reasonably close contact, not all the parents need to re-excrete to ensure virus transmission to all the offspring. Once one clinical case has occurred, virus dissemination will be greatly increased. Nevertheless, such a system, which tends to be seen particularly in cat colonies, is not ideal, since the timing of shedding in relation to the presence or absence of maternal antibody may be less than perfect. Severe clinical disease may then ensue and mortality and chronic sequelae may be significant, thus jeopardizing the host's and therefore the virus's survival into the next generation.

Another useful tool for the virus epidemiologically is the increased likelihood of shedding after a change in living conditions, a situation which has been demonstrated particularly in cats. Thus if an animal is driven from its territory or family group, then it is likely to re-excrete virus in its new surroundings and may therefore infect another population. By this means the virus has a mechanism for increasing its chances of horizontal spread.

In conclusion, observations on natural host-virus systems may tell us more about the biological significance of latency, and indeed, more about the host-virus relationship in general. Perhaps if most natural infections occur in young animals when they are losing their passive immunity, this should be the age at which both the mechanisms for establishing and controlling latency should be examined. It should be interesting to compare the frequency of recrudescent disease in people or animals that have experienced asymptomatic primary infection at an early age under cover of passive immunity, with those who experienced later symptomatic infections. Finally perhaps hormone influences on re-excretion rates and recrudescent disease should be examined in more detail.

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GENETIC RESISTANCE TO HERPES SIMPLEX VIRUS-1 INFECTIONS AND THE IMMUNE SYSTEM

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ABSTRACT

C57BL/6 mice are genetically resistant to infection with herpes simplex virus type 1 (Lopez, 1975). Mice of this strain eliminate the infecting virus from the liver and spleen after intraperitoneal infection. Mice of genetically sensitive strains die of the infection. The genetically resistant C57BL/6 mice do not develop a herpesvirus infection when the herpes simplex virus-1 is inoculated into the nose and eyes and, at low virus doses, also intracerebrally. We suggest that the cell system involved in the protection of the C57BL/6 mice against the virulent virus is the reticuloendothelial system (RES: Kupffer cells in the liver, dendritic cells in the spleen, Langerhans cells in the skin, etc.). These cells are produced in the bone marrow and migrate to different organs to become specific organ-associated macrophages. It is hypothesized that in the genetically resistant C57BL/6 mice, the two genes which confer genetic resistance are involved in the elimination of the virus. The RES is involved in the presentation of antigens to the lymphocytes of the immune system, and thus control part of the immune response. Destruction of the RES by a herpesvirus might lead to an incomplete immune response.

In a review of genetic determinants of virus susceptibility, it was stated by Brinton and Nathanson (1981) that

> "In focusing on host genetics, it is important to keep in mind that the severity of disease is the result of the interaction between host resistance genes, virus virulence genes, and host defense systems. Both host and viral genes play an important role in maintaining a balance which ensures the continued survival of the host and the virus under natural conditions"(p. 116, M.A. Brinton and N. Nathanson. Genetic determinants of virus susceptibility: epidemiologic implications of murine models. Epidemiologic Reviews 3:115-139, 1981.)

Herpesviruses which have been isolated from many vertebrate hosts follow the balance indicated above, ensuring a virus-host interaction which allows survival of the host and virus in most instances. Herpes simplex virus (HSV) type 1 infections in humans are rarely fatal, but the virus becomes latent in the nervous tissues. Even when the virus is reactivated

it moves with the axonal flow in the neuron in which it is reactivated in the direction of the skin, and not in the direction of the central nervous system (CNS). Nevertheless, damage to the immune system as a result of a genetic disorder or immune suppression by drugs, leads to the uncontrolled spread of the virus and to a lethal generalized HSV-1 infection (Nahmias et al., 1981). Thus, the balance between a localized HSV-1 infection and a disseminated one is dependent on the immune system which is involved in the control of the incoming virulent virus. Regarding Epstein-Barr virus (EBV), another human herpesvirus pathogen, individuals respond differently to infection: a) most individuals are infected during childhood and develop a positive immune response; b) seronegative young adults develop infectious mononucleosis (IM), resulting in proliferation of T cells; and c) in certain populations, the virus leads to the development of Burkitt's lymphoma (BL) (e.g. Africans in Uganda) and nasopharyngeal carcinoma (NPC) (e.g. Chinese in south China, and North Africans). In all EBV-infected individuals with IM, BL and NPC, antibodies to viral antigens and enzymes are produced by the immune system. Nevertheless, the virus is capable of exerting its ill effects on only a few individuals in the EBV-infected populations.

Another known possibility is the transfer of a herpesvirus from a host which is resistant to the virus to a highly sensitive host. A good example is monkey B virus, which does not harm the monkey host but is highly pathogenic for humans. This example indicates that a herpesvirus infecting a sensitive host might have the ability to abrogate the immune defense system and, in its absence, the infected host is unable to defend itself. Also, injection of EBV into owl monkeys leads to the development of lymphomas, suggesting that this monkey is defenseless against this herpesvirus.

Studies on the sensitivity of various laboratory mouse strains to viruses (reviewed by Brinton and Nathanson, 1981) revealed differences in their sensitivity to viruses. The inbred strain of black mice (designated C67BL/6) is resistant to infection with many viruses: a) to picornaviruses which cause diabetes in a sensitive mouse strain SWRJ (Onodera et al., 1978); b) to murine leukemia virus (Lilly, 1970; Axelrod and Steeves, 1964; Blank et al., 1976); c) to Friend leukemia virus (recovery from splenomegaly), while mouse strain Balb/c is highly sensitive (Doig and Chesebro, 1979); d) to polyoma virus which causes tumors and runting in the AKR mouse strain (Chang and Hildemann, 1964); e) to ectromelia (mousepox) which infects CBA mice (Myers et al., 1954); and f) to herpes simplex virus type 1 which

causes encephalitis in many other mouse strains (Lopez, 1975).

The studies on the genetic resistance of C57BL mice to HSV were reviewed by Lopez (1981). Intraperitoneal (i.p.) injection of virulent HSV-1 into C57BL/6 mice resulted in the disappearance of the virus, namely, the host was able to overcome the infecting virus (Lopez, 1975; Kirchner et al., 1978). This phenomenon was quantitated by Lopez et al. (1980), who reported that after i.p. injection of HSV-1 into C57BL/6 mice, the virus was found in the liver and spleen at day 1 after infection. At days 3 and 5, no virus was detected in livers and spleens of the infected mice. A significant further finding reported by Lopez et al. (1980) was the observation that when BDF1 mice, which are a cross between C57BL/6 mice and DBA/2 mice, were treated with the bone marrow-seeking radionuclide ^{89}Strontium (^{89}Sr), the invading virus was present in the liver and spleen at 1, 3 and 5 days post-infection. The virus invaded the spinal cord and the CNS, and the mice died of viral encephalitis, whereas nonirradiated mice overcame the virus infection. These studies led Lopez et al. (1980) to suggest that bone marrow-derived natural killer cells are involved in the ability of genetically resistant mice to overcome the virus infection. Kirchner et al. (1982) maintain that endogenously produced interferon plays a critical role in the genetic resistance to HSV-1 infection in the mouse, and not natural killer cells.

Another cell type that might be involved in genetic resistance is marrow-dependent macrophages (Stevens and Cook, 1971). Lopez and Dudas (1979) reported that peritoneal macrophages from C57BL/6 mice restrict HSV-1 replication significantly better than macrophages from susceptible mice. However, this function is not directly related to resistance, since macrophages from resistant F_1 mice failed to restrict HSV-1 replication.

Studies by Lopez (1980) on progeny of the F-2 cross using C57BL/6 x A/J (susceptible) F_1 mice suggested that two major loci were responsible for resistance. Studies with congenic mice showed that genes within the H-2 locus did not influence resistance or susceptibility to HSV-1.

Resistance and sensitivity of mouse strains to HSV-1

The ability of C57BL/6 mice to restrict HSV-1 infection and the mechanism of genetic resistance was studied in our laboratory, with special attention to the response of C57BL/6 mice to HSV-1 infection in the nose, eyes and CNS.

Various strains of mice were injected i.p. and intracerebrally (i.c.) with HSV-1 (TK^+ large plaque NIH strain, isolated in our laboratory; Gordon

et al., 1983; Ben-Hur et al., 1983). Table 1 shows that two of the three mouse strains (Sabra and AKR) were highly sensitive, whereas C57BL/6 was highly resistant to i.p. virus injection, as reported by Lopez (1975).

Table 1. Genetic resistance and sensitivity of mouse strains to infection with herpes simplex virus-1.

Mouse strain	Age	Irradiation	Mode of injection	
			Intraperitoneal (I.P.)	Intracerebral (I.C.)
Sabra	4 weeks	None	$10^{2.85}$ [a]	10^{0}
AKR	4 weeks	None	$10^{3.7}$	10^{0}
C57BL/6	4 weeks	None	10^{6}	$10^{3.1}$
C57BL/6	4 weeks	500 rad [b]	Highly sensitive	ND [c]
C57BL/6	<4 weeks	None	Highly sensitive	ND

[a] Virus titer (pfu/mouse) leading to death of 50% of the infected mice

[b] Total body irradiation of mice

[c] Not done

The same was true when the virus was injected i.c.. Whole body radiation (500 rad) resulted in the abrogation of the resistance of C57BL/6 mice to HSV-1. Also, C57BL/6 mice younger than four weeks were found to be sensitive to HSV-1 infection. This suggests that "genetic resistance" to HSV-1 gradually develops during the first four weeks of the mouse life cycle in parallel to the development of the immune system.

Further studies revealed that C57BL/6 mice are resistant to HSV-1 infection in the nose. This mode of infection enables the virus to penetrate the olfactory bulb and migrate to the CNS (Fig. 1). Infection of C57BL/6 mice by inoculation of the virus onto the cornea resulted in the appearance of the virus in the trigeminal ganglion on day 5 and disappearance of the virus from the ganglia afterwards (T. Ben-Hur and Y. Becker, to be published).

These results have confirmed and extended the studies by Lopez (1975, 1981) and indicate that the genetic resistance of the C57BL/6 mouse is expressed not only in the liver and spleen, but also in the eyes, trigeminal

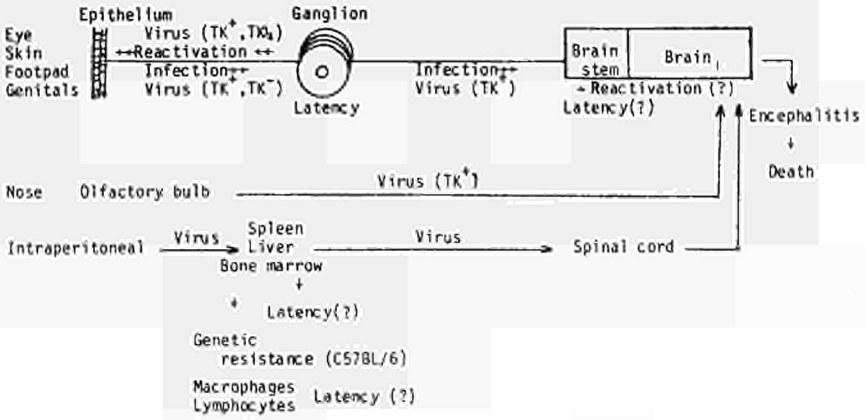

Fig. 1 Routes of infection by herpes simplex virus type 1 in the mouse. The virus isolates from an N.I.H. strain in our laboratory differ in their thymidine kinase (TK) activity and their virulence for mice: TK^+, active TK gene; $TK^¼$, 25% of the TK activity of the TK^+ strain; TK^-, no TK activity (Gordon et al., 1983; Ben-Hur et al.,1983).

ganglia and CNS.

Nature of the cell system which might be involved in genetic resistance

Lopez (1981), in discussing the cells which might be involved in the genetic resistance to HSV-1 favored the possibility of natural killer cell involvement in the restriction of HSV-1 infection, although the role of macrophages was also mentioned. The cell system which we favor as having a major role in the genetic resistance to HSV-1 is the reticuloendothelial system of tissue-specific macrophages that are of bone marrow origin and express the Ia antigen (mouse immune response gene-associated antigen) on the cell surface. These cells in the C57BL/6 mouse interact with the invading viruses and, due to their resistance genes, they are able to restrict virus replication. These cells also present the foreign (viral) antigens to lymphocytes of the immune system which eventually produce specific

Table 2. Possible role of tissue-specific reticuloendothelial cells (in liver and spleen) in the prevention of herpes simplex virus-1 infection in genetically resistant mice

Mode of HSV-1 infection	Tissue	Mice	
	Reticuloendothelial system*	Genetically resistant (C57BL/6)	Genetically sensitive (AKR)
Intraperitoneal	Liver: Kupffer cells	Virus destruction**	Virus replication**
	Spleen: Denritic cells and/or macrophages	Virus destruction**	Virus replication**
Intradermal	Skin: Langerhans cells	Not known	Not known
Intracerebral	Nervous tissue, brain and ganglia: Microglea cells (?)	Resistant to virus dose which kills sensitive mice. Sensitive to IC injection of $10^{3.1}$ pfu/mouse	Virus replicates and kills (LD_{50} = 1 pfu)

* Cell types: Members of the reticuloendothelial system which are antigen presenting cells expressing Ia antigen on their cell surface and originate in the bone marrow.

** From C. Lopez, R. Ryshke and M. Bennett, Infection and Immunity 28:1028-1032, 1980.

immunoglobulins (Schwartz et al., 1978).

We would like to suggest that Kupffer cells in the liver (Berzofsky et al., 1979), Langerhans cells in the skin (Stingl et al., 1978),dendritic cells in the spleen (Steinman and Cohen, 1973), and microglea cells in the CNS which are of bone marrow origin (Ting et al., 1983) might be involved in the defence mechanism (genetic resistance) against HSV-1. Table 2 presents the hypothesis that reticuloendothelial cells might be involved in the genetic resistance to HSV-1. Namely, it is suggested that the two genes involved in the genetic resistance are expressed in the macrophages of the reticuloendothelial system, and the gene products enhance the ability of the Kupffer and dendritic cells to destroy and eliminate the virus. This might be the explanation for the results reported by Lopez et al. (1975). Kupffer and dendritic cells most probably present the viral antigens to the T and B lymphocytes, respectively, for antibody induction. Viral antigens associated with such macrophages are far more immunogenic than free viral antigens. Thus, Kupffer and dendritic cells most probably are able to destroy the infecting virus and are efficient in stimulating the immune response.

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LOCAL IMMUNITY TO HERPESVIRUSES

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Our present understanding of local immune systems has developed largely out of the discovery of IgA (Heremans et al 1959) and the subsequent observations that it was the predominant immunoglobulin in milk and other exocrine secretions (Hanson, 1961; Tomasi et al 1965). These findings formed the basis of the concept of a local immune system mediated by IgA antibody which is stimulated by, and which controls events at mucosal surfaces. These ideas were strengthened by the discovery that precursor IgA lymphoblasts show distinct migratory pathways that are relatively independent of the spleen and peripheral lymph nodes (for review see Bienenstock & Befus, 1980). Further support for this concept came from the observations of Bienenstock (1974) who showed that similar lymphoid follicles could be found at different mucosal surfaces and that lymphoblasts isolated from them showed similar homing characteristics (Rudzik et al 1975). Although the precise nature of a common mucosal immune system has been questioned (Spencer & Hill 1984) there is no doubt of the independence of immune mechanisms that operate at mucosal surfaces as opposed to those found systemically.

In general herpesviruses are presented at the mucosal surfaces of the respiratory and reproductive tracts. It is therefore appropriate to review the immune mechanisms that operate at these sites in an attempt to understand how they might be stimulated to afford protection.

a) RESPIRATORY TRACT

Defence mechanisms that operate in the lung are like others, multifactorial involving both non-specific and specific acquired elements. In the former category one can list anatomical disposition within the respiratory tract as results from the anatomy of the tract, the mucociliary apparatus, non-specific cellular mechanisms and humoral factors such as lysozymes, lactoferrin, surfactants, "normal flora", complement and interferon. Whilst a number of these are of no significance against viruses others do provide a first line of defence.

Immunoglobulins, G, A, M and E have all been detected in respiratory fluids. Dimeric locally synthesised and secretory IgA, predominates most dramatically in the upper tract. Although a number of effector functions have been ascribed to IgA including viral neutralization (Ogra et al 1968)

opsonic activity (Reynolds et al 1975) antibody dependant cell mediated cytotoxicity (Lowell et al 1980) (for review see McDermott et al 1982), it has also been suggested that its principal role is anti-inflammatory, preventing immunogenic material from either being absorbed across mucosal membranes (Stokes et al 1975) or from stimulating other immune mechanisms such as chemotaxis (Van Epps et al 1976) and complement activation (Griffus & Bertram 1977).

IgG also occurs at relatively high concentrations in pulmonary secretions derived from both local production and serum transudation. The precise role of IgG in mucosal secretions is unclear, but in the lower respiratory tract where its levels relative to other immunoglobulins are highest, and approaches those found in serum (Kaltreider and Chan 1976) it may well have a similar virus neutralisation role to that ascribed for serum IgG.

IgM is present at only low concentrations in respiratory secretions and little is known of its significance at this site except in IgA deficient individuals where it may take over (Baklier & Brandtzaeg 1976). Bronchioalveolar washings have also been shown to contain IgE (Deuschl & Johansson 1974) but no significance has been attached to it with respect to viral immunity. The recent description of high levels of IgD in milk (Olson & Leslie 1982; Brandtzaeg 1983) would indicate that this immunoglobulin may also have a role in mucosal immunity, at present it remains undetermined.

The airspaces within the bronchi, bronchioles and alveoli contain significant free cell populations. In all species studied the predominant cell type isolated from bronchioalveolar washings of healthy lungs are macrophages which may comprise up to 95% of the total cell population recovered. Polymorphs and lymphocytes may also be present in significant numbers and in washing taken from infected lungs the former may take over as the predominant cell type.

The alveolar macrophage is primarily bone marrow derived although there is evidence of local proliferation. Whilst their main activity is the non-specific ingestion of particles in the lung, their phagocytic activity is markedly enhanced by the presence of specific antibody. Interestingly viral infection may interfere with this activity Jakab et al 1980). The relative importance of alveolar macrophages in the interaction and induction of sensitised T-lymphocytes as opposed to those at other respiratory sites (i.e. BALT, draining lymph nodes, etc.) remains to be determined.

Fractionation studies of lymphocytes from bronchioalveolar washings indicate T-cells are the major cell type although significant numbers of B-

and null cells have been found. Of the B-cells, IgA is the predominant surface immunoglobulin (Davis et al 1980), and most cells synthesised this immunoglobulin isotype. The T-cell population is heterogenous and includes both those capable of eliciting delayed type hypersensitivity reaction and specific cytotoxicity. Natural killer (NK) cell activity has also been reported within the free cell population in the lung.

The cells found in bronchioalveolar washings are but a small portion of the lymphoid population associated with the lung, that includes the bronchus associated lymphoid tissue (BALT) and the palatine and nasopharyngeal tonsils. Detailed descriptions of the lymphoepithelium that overlie the lymphoid follicles that together make up BALT have been provided (see McDermott et al 1982). Functionally they have an important role in uptake of antigen taken into the lung, and its presentation to professional immune cells. By analogy with the gut associated lymphoid tissue it is likely that such presentation leads to the generation of IgA (+ ? IgG) plasmacyte precursors and regulator T-cells, that can migrate and home to both the lung lamina propria and other mucosal sites.

The tonsils are ideally placed and suited for trapping inhaled antigens, having an epithelium capable of absorbing antigen. Although they contain both T and B lymphocytes it is not clear how they are involved in immunity. Interestingly we have recently shown that following instillation of feline viral rhinotracheitis virus into the nose of cats, the virus may be detected in the tonsil, and that in immune animals although the virus can apparently get into the tonsil it is rapidly killed (Cocker et al 1984).

b) REPRODUCTIVE TRACT (Female)

Histologically the reproductive tract is a mucous membrane, however unlike the respiratory and gastrointestinal tract it is not exposed to the continuous antigenic challenge. Despite this the vagina does have a resident microflora although the uterus remains sterile. Like the other mucosal surfaces a number of non-specific defence mechanisms operate. These include the structure of the cervix, mucus and anti-microbial proteins.

The immunoglobulins have been detected in the reproductive tract fluids of a number of species, although there is considerable variation between species and with the different levels of the tract. In general though follicular fluids tend to be dependent on serum and thus the immunoglobulins present reflect their serum concentrations and molecule size. Further down the tract in the uterus again IgG tends to predominate, but in a number of species this has been shown to be of local origin. Thus there would appear

to be a decrease in the dependence upon serum transudation the further the tract is descended (Widders et al 1984) in the cow (Corbeil et al 1976) IgA predominates in the vagina.

The importance of cellular elements in the defence of the reproductive tract is less well defined. However both lymphocytes and polymorphs can be isolated in washings from the tract.

THE IMMUNE RESPONSE TO LOCAL IMMUNISATION

It is a generally accepted concept that local application of antigen is most effective in stimulating protective immune responses. For example intranasal instillation of influenza virus resulted in ten times as much secretory antibody in nasal secretions as was produced following sub-cutaneous immunisation (Fazekas de St. Groth et al 1950).

Soluble antigens presented to the Bronchus associated lymphoid tissue may be taken up into the lymphoepithelium by pinosytosis (Tenner-Racz et al 1979) in a manner similar to that shown for M-cells of Peyer's patches (Owen, 1977). Such antigen may then be transferred to the follicle where there is an abundance of lymphocytes. The response elicited by dead antigens presented to mucosal surfaces is highly dose dependant, the greatest responses requiring very large doses of antigen (for review see Newby & Stokes 1984). Far greater responses may be achieved with the use of live organisms at doses sufficiently large to promote multiplication. Whilst early studies questioned whether secondary response could be elicited by mucosal stimulation, it is clear from studies in the gut that an anamnestic can be elicited (Andrew & Hall 1982). Interestingly there is evidence from influenza virus infections that the dose and frequency of immunization influences whether a secondary response occurred (Shwartsman & Zykov, 1976). Further rabbits immunised with live Shigella produced a good secretory IgA antibody response on challenge 60 days later, whilst those fed the dead antigen showed no evidence of memory (Keren et al 1982).

A common feature of antigen presentation at mucosal surface is the development of hyporesponsiveness. It has been postulated that these mechanisms are important in preventing overreaction (allergy) to food antigens (for review see Newby & Stokes 1984). Such events are rarely induced to microbial antigens (Stokes et al 1979), but it has been shown that whilst oral immunisation of mice with living reovirus stimulates a strong delayed type hypersensitivity response whilst using the dead virus produces a specific immunologic tolerance to the viral antigens (Rubin et al 1981).

Respiratory infection with a variety of viruses produces a local secretory antibody response that is primarily IgA; (Blandford & Heath, 1974) and its presence may indicate protection. Little data is available on the response to herpesviruses, but by analogy to studies with other viruses it is likely that whilst serum antibody may protect in the lower airways, locally produced antibody will be protective in the upper respiratory tract (Ramphal et al 1979; Blandford & Heath 1974).

It is possible that cell mediated immune reactions may provide protection. Like humoral immunity, local CMI may best be stimulated by local application of antigen (Nash & Holle, 1973). The importance of cells in the respiratory tract has been emphasised by studies of calves infected with bovine herpes. Whilst the addition of blood lymphocytes with or without serum had a slight inhibition of viral replication (BHV-1) in bovine tissue culture cells, cells collected from bronchio-alveolar washings could markedly inhibit the virus (Bouffard et al 1982). Similarly nasal leukocytes taken from cattle vaccinated intranasally with temperature sensitive mutant IBR were able to protect from cytopathological changes whilst those that received the virus intramuscularly were not (Gerber et al 1977). The appearance of interferon in nasal secretions has also been associated with local immunisation (Todd et al 1972) where its appearance it was suggested coincided with protection.

That mucosal stimulation can be effective in generating protection from subsequent infection by a herpesvirus is thus established. For example oral vaccination with Herpes simplex type I can enhance the protection against intravaginal challenge with Herpes simplex type II (Sturn & Schneweis 1978). Whilst much has been written about how protection may be afforded systemically (Onions 1982; Rouse & Horoho✓ 1984) the mechanisms that operate against herpesviruses at mucosal surfaces remain unclear. Since both local humoral and cellular immune factors operate independently of systemic immunity it remains important to determine first how protection is afforded and secondly how best it may be stimulated.

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SUMMARY OF SESSION TWO.

THE IMMUNE SYSTEM IN THE DEFENCE AGAINST HERPESVIRUSES.

by B.T. ROUSE and C.R. STOKES.

The session, for convenience, may be considered in three parts, covering the biology of herpesvirus infections, genetic resistance and acquired immunity.

In the first GASKELL describes two herpesvirus infections, Felid herpesvirus 1 (FHV 1 - formerly known as feline viral rhinotracheitis (FVR) virus) and Pigeon herpesvirus 1 (PHV-1). The advantage of these natural host-virus systems for the study of latency and re-activation by natural stimuli were emphasized. For example the post-partum shedding of virus from carrier queens and the establishment of a latent carrier state in the kittens whilst under the cover of passive immunity, was used to indicate the biological role of latency.

BECKER described how certain inbred strains of mice are resistant to the effects of virus infection. Such genetic resistance being a feature of C_{57} black/6 mice which are resistant to a number of viruses. Following I.P. injection virus could be detected in the liver and spleen on day 1 but by day 3-5 it was completely eliminated. Cellular depletion studies indicated that the responsible cells are sensitive to ^{89}Strontium, whole body irradiation (500 rads) and are probably bone marrow derived. Young mice (less than 4/52) are sensitive. Interestingly the route of "infection" with virus is critical, whilst mice are resistant to I.P. infection following eye or nose infection the virus can be found in the ganglion.

The importance of route of infection was also illustrated by the two other talks. ROUSE emphasized the importance of natural defence mechanisms against initial infections and indicated the importance of natural killer cells, macrophages and interferons in this role. In considering local immunity to herpesviruses, STOKES took up this point and describes how non-specific defence mechanisms operate at mucosal surfaces (mucus, cilia, interferon, macrophages, physical structure) and are essential for the handling of viruses at their main sites of entry namely the respiratory and reproductive tracts. ROUSE focused on T-lymphocyte mediated immunity since such mechanisms have been shown to predominate over antibody in systemic immunity to herpesviruses. The immuno-pathological lesions associated with such responses were also discussed. The immune mechanisms that operate at the mucosal surfaces of the respiratory and reproductive tracts were illustrated (STOKES). The independence of local and systemic immune systems was re-emphasized and the importance of local immunity in the protection from experimental herpesvirus challenge was discussed. Finally the problems associated with the development of safe mucosal vaccines were indicated.

SESSION 3

IMMUNE RESPONSE TO HERPESVIRUSES IN CATTLE

Chairman : G. CASTRUCCI

Co-chairman : E. THIRY

FORMAL ANALYSIS OF THE INTERACTION BETWEEN THE SPECIFIC IMMUNE RESPONSE OF CATTLE AND *BOVINE HERPESVIRUS 1*

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ABSTRACT

A formalisation of infectious bovine rhinotracheitis virus (*Bovine herpesvirus 1*) latent infection was built by logical analysis. The formal description deals essentially with the events that appear after primary infection, reactivation or vaccination with inactivated or attenuated vaccines, in a virgin animal or a latently infected one. It consists of a set of five logical equations defining five functions: V (viral multiplication); R (development of reactivation of latent virus); A (development of an immune response); G (establishment of the viral genome); M (development of a memory of a first immune response). Five internal variables (v; r; a; g; m) are associated with the five logical functions. In addition, there are four input variables: i_v (infection with a virulent virus); i_a (infection with an attenuated virus); v_i (injection of inactivated virus); d (treatment with dexamethasone or any exogenous or endogenous stimulus able to provoke reactivation). Time delays of appearance or disappearance are given for each internal variables.

INTRODUCTION

The infection of cattle with *Bovine herpesvirus 1* (BHV 1) provokes two major diseases: infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV) (Gibbs and Rweyemamu, 1977). After recovery from primary infection, the virus persists in the animal in a latent form: it is no more detectable by means of conventional virological methods.

Latency, a biological property of great epidemiological importance, is shared by the herpesviridae (Honess and Watson, 1977). In latently infected animals, the virus is occasionally reactivated; it can be re-excreted without any clinical sign and, by this way, transmitted silently to virgin animals. Latency also allows BHV 1 to persist in a restricted herd without introduction of exogenous virus.

BHV 1 latency is well documented and it is a good model for several reasons: the phenomenon of latency can be studied in the proper species where it appears and in a naturally fixed situation (Pastoret et al., 1982). In addition, BHV 1 can be experimentally reactivated by the use of glucocorticoids (dexamethasone). The re-excretion of BHV 1 is modulated by

the level of the specific immunity, and, when this level is above a certain threshold, reactivation can occur without re-excretion of virions (Pastoret et al., 1980).

The aim of this work is not to provide a detailed model of BHV 1 latent infection: not enough is known so far about the mechanisms. More modestly, we wish to present a formal description of our present view of the system, with special emphasis on the conditions of viral re-excretion.

A first justification is that, as in many other fields, the steady accumulation of facts makes it increasingly difficult to have a global view of the subject: a formal description may help although it is necessarily over-simplified. The main objective, however, is to provide a way to check the consistency of the views we may have on such a complex system. Besides the essential point of self-consistency, models are usually built to account for a small number of selected features of a system, but formal analysis almost invariably shows up implications which had not been considered during the modelling. Some of them will be consistent with already known facts. Others are "predictions" which will have to be submitted to the test of experience.

As in many other cases, a quantitative description of the situation would only serve to give the impression that we know more on the system than we really do. We would be very satisfied with a qualitative, if coherent, description. This is why we turned to a logical formalisation, i.e. a description which uses variables with a limited number of values (typically, two only: 0 and 1). More specifically, we use the method described by Thomas (1973; 1979). It has become clear that the essential dynamical aspects of complex systems are preserved by a proper logical description, in spite of its apparently caricatural character.

DESCRIPTION OF BHV 1 LATENT INFECTION

BHV 1 infection

The pathology of BHV 1 infection is largely due to respiratory infection; the virus is usually transmitted by direct contact from animal to animal. After a primary infection, virus replicates in epithelial cells of the anterior respiratory tract. Thus during a primary infection, when virus is replicating intensively, a large amount of virions is present in nasal exudates (Pastoret et al., 1979). During this period of intense virus multiplication, the animal is highly contagious for its surroundings during

several days. The same is true when the animal shows the genital form (IPV), but in this case, the transmission is venereal.

Animals recover within two weeks, except when bacterial superinfection occurs (Gibbs and Rweyemamu, 1977).

The latency of BHV 1

After recovery from the primary infection, BHV 1 infectious particles are no more detected in nasal mucus or vaginal samples and, moreover, the animal is immune. As already mentioned, the virus persists in the host in a latent form. The conditions required for the establishment of latency are still under investigation: especially, it is not known if the dose of virus to which the animal is exposed during primary infection and its subsequent multiplication is important for the establishment of latency. It is not yet known to which extent the immune stage of the animal following a primary infection influences the ability of another strain to establish latency in the same animal.

Nevertheless, the latency of BHV 1 has already been investigated in several models: the most extensively studied models involved attenuated vaccine strains. In this case, it is established that: (1) attenuated strains can remain in a latent state (Pastoret et al., 1980) and, for the thermosensitive (ts) strain (Zygraïch et al., 1974), even after usual vaccination procedure (Nettleton et al., 1984); (2) vaccination by attenuated or inactivated vaccines does not prevent the further installation of challenge wild virus in a latent form (Darcel le Q. and Dorward, 1975; Nettleton et al., 1984); (3) primary infection with a virulent strain seems to prevent latency of a reinfecting attenuated strain (Thiry et al., 1985a). All animals then carry in a latent form the virus after primary infection; in addition, in spite of vaccination, a superinfecting virus will induce latency if it is virulent.

BHV 1 reactivation and re-excretion

Reactivation of BHV 1 can occur by means of several endogenous or exogenous stimuli, including the injection of glucocorticoids especially dexamethasone (Snowdon, 1965; Sheffy and Davies, 1972). The mechanism of BHV 1 reactivation is still unknown. The reactivation of latent virus may be followed by re-excretion of infectious particles (Snowdon, 1965) and non-infectious particles (Pastoret et al., 1979). In other words,

reactivation without re-excretion of virions is an encountered situation (Thiry et al., 1983a).

In fact, the re-excretion of latent BHV 1 is modulated by the level of specific immunity, which, in turn, is dependent on the schedule of previous infections, reactivations or vaccinations, and whether the immune response was induced by the multiplication of a wild-type or an attenuated virus (Straub and Wagner, 1977; Rossi and Kiesel, 1982).

The sequence of events may be interpreted as follows (Pastoret et al., 1980, 1984): after a primary infection, the animal is able, by its immune system, to prevent the clinical effects of a reinfection with a virulent strain but cannot properly control an episode of re-excretion (Davies and Carmichael, 1973). The first viral reactivation with or without re-excretion produces an increase of the specific immune status and of the efficiency of some immune mechanisms. The animal is therefore able to control a further reactivation better and for a longer time and no re-excretion occurs.

Animals which, under experimental conditions (e.g. dexamethasone treatment), excrete the highest level of virus after reactivation are those with the lowest specific immune response. This observation supports the view that re-excretion is influenced by the specific immune stage of the animal (Pastoret et al., 1979, 1980).

The occurrence of spontaneous viral reactivation and re-excretion is not well documented. It is studied by the detection of re-excretion of BHV 1 virions and of a rise in specific antibodies. The spontaneous re-excretion of field strains of BHV 1 occurs in experimental animals and in closed herds at least until one year after infection (Snowdon, 1965; Hyland et al., 1975; Bitsch, 1975): this is reported for both the genital and the respiratory infections. The spontaneous re-excretion of attenuated strains, administered intranasally, is probably a rare event (Thiry et al., 1983c).

Reactivation and re-excretion of BHV 1 can be provoked repeatedly in the same animal by dexamethasone treatment (Pastoret et al., 1979).

METHODOLOGY

Kinetic logic

The method known as kinetic logic (Thomas, 1973) has been described in detail elsewhere (Thomas, 1979; 1984). In short, to each of the elements

whose decription seems crucial to us we associate both a logical variable (a, b, c, ...) whose value (1 or 0) tells whether the element is or is not present at a significant level, and a logical function (A, B, C, ...) which tells whether the element is being produced at a significant rate.

For instance, in a genetic system, the logical value of a variable tells whether the gene product is present, the logical value of the corresponding function tells whether the gene is on . More generally, our logical variables usually describe concentrations while our functions describe rate of production. It must be emphasized that a=0 (product "absent") does not mean that the product is completely absent; it means that the product is below its threshold level.

In our context, variable v (for instance) takes the value 1 when virions are actually present and the corresponding function V takes the value 1 when virions are being produced. In a state of regime, v and V have the same value: virions have not been produced (V=0) for a long time and there are no more virions present (v=0); or virions have been produced (V=1) for some time and are present (v=1). However, when the value of V has recently changed, v and V may have different values: virions are being produced but they are not yet present at a detectable level (v=0); or virions have stopped being produced (V=0) but they are still present (v=1) (figure 1).

There is thus a rather simple temporal relation between a variable and the corresponding function. Let us start from a state of regime in which a function and its variable have the same logical value. If a signal changes the value of the function (i.e. switches it on or off), the variable adopts the new value of the function, but only after a characteristic time delay; if function V has been switched on, one must wait from a time t_v before virions appear; if function V has been switched off, one must wait for a time $t_{\bar{v}}$ before virions disappear. There is no reason why the "on" and "off" delays should be the same; in practice, they are often very different. Note that during the transient period in which a function and the corresponding variables have different values, the logical value of the variable serves as a memory of the preceding value of the function.

As shown in detail elsewhere (Thomas, 1984), we describe a system by a set of logical equations which relate the value of each function to the values of the relevant variables; more concretely, the logical equations tell in what conditions each function is on or off, depending on the

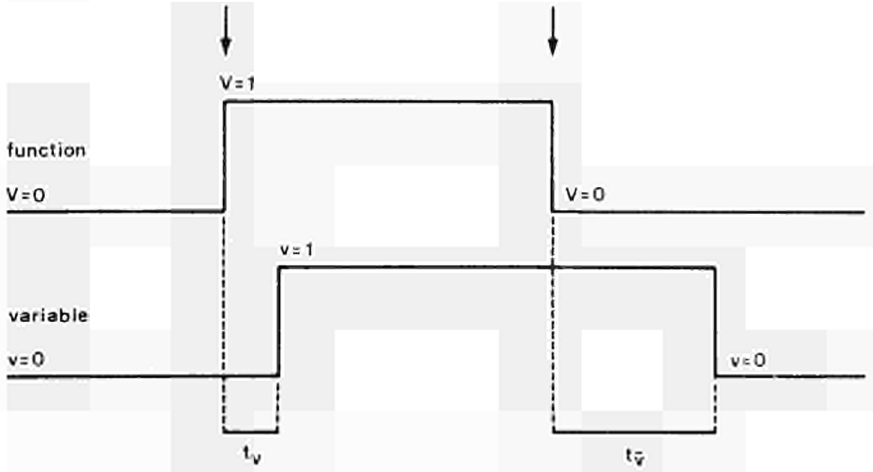

Fig. 1 The logical value of variable v functions as a memory of the preceding value of function V.
The arrows indicate that a signal has changed the value of V (more concretely, switched on or off the synthesis of virions). This operates as an order to change the value v (more concretely, to make virions appear or disappear, according to the case). But this order is executed only after a suitable delay, t_v or $t_{\bar{v}}$ depending on whether the function is switched on or off. If a counterorder takes place before the order has been executed, we reason as if the order had not been given.

presence or absence of the relevant elements. A logical equation of the form A = f (a, b, c, ...) means that function A is on (A has the value 1) if the values of variables a, b, c, ... are such that (a, b, c) = 1. We use the classical connectives:

a.b (or ab) means a AND b (logical product)

a + b means a OR (inclusive) b (logical sum)

$\bar{a}$ means NOT a (logical complement)

For instance, $A = a\bar{b} + c$ means that function A has the value 1 if a (and not b) or c, or both, have the value 1. These logical equations resemble the differential equations used in chemical kinetics in the sense that both relate rates of synthesis to concentrations: and this is how the time is (implicitely) included in our logical equations.

From these logical equations, one can build a state table, which gives the values of the functions for each combination of values of the variables. Note that in addition to the internal variables described so far, one has to consider input variables. While, as already mentioned, the value of an internal variable is a delayed effect of the value of the

corresponding function, the values of input variables can typically be changed at will. For instance, one can decide to submit a "virgin" animal to infection with BHV 1 (i), or reactivate the latent virus using an appropriate stimulus (d); i and d are input variables.

From the state tables, one can derive all the sequences of states (pathways) of the system. Which sequence(s) is (are) followed depends on the relative values of time delays. Which are involved in the choices between sequences of states, and how, are determined by a subsequent analysis.

A formal description of the BHV 1 infection

Various choices of functions and variables are conceivable. Those we finally chose are shown in table 1.

TABLE 1 Functions and variables

Functions :	Variables :
V : development of viral multiplication	v : presence of virions
R : development of reactivation of the latent virus	r : effective reactivation of the latent virus
A : development of an immune response	a : presence of specific active immunity
G : establishment of the viral genome	g : presence and persistence of the viral genome
M : development of a memory of a first immune response	m : presence of a memory of a first immune response

Now, a few comments on this table. Function V represents viral multiplication . The associated variable v refers to the presence of mature virions. As regards the immune response we chose to describe it simply by the presence (a) or absence ($\bar{a}$) of active specific immunity directed against the virus, and by the presence (m) or absence ($\bar{m}$) of a memory of an earlier response. The logical functions associated with these variables are repectively, A, which refers to the development or maintenance of the response, and M, which refers to the establishment or maintenance of a memory of the response. It may be noticed that in this symbolism, primary and secondary (or more generally, anamnestic) responses are represented by the same variable a. However, the value of m tells us whether one deals with a primary (m=0) or a secondary (m=1) response, and depending on the case, the time delays are very different (table 2). In other words, instead of explicitely using distinct variables and functions to describe the

primary and secondary responses, we distinguish between the two situations by the values of the time delays (a secondary response develops faster and persists longer).

The reason for introducing variable m is that while value, 0 or 1, of variable a tells whether specific immunity is above or below a protective threshold now, we need a variable whose value tells whether it is or has been present. Clearly, if one wrote M = a, function M would take the value 1 some time after the appearance of immunity, but it would drop back to 0 after immunity has disappeared (see again fig. 1 for the time dependence between a function and its associated variable). However, if M = a + m (its means a or m), once m has taken the value 1, both M and m will keep this value. More generally, the simple logical structure X = ... + x ensures that once x has reached the value 1 both X and x are blocked for ever at this value. Similarly, we have to express that once an animal has been successfully infected with BHV 1, the viral genome will persist indefinitely. This can be written: G = v + g. Most of the time, the virus will then remain latent, but following appropriate treatment, e.g. dexamethasone treatment (d), the virus is reactivated. When we write : R = gd, we implicitely assume that independently of the above-mentioned effect of immunity on viral multiplication, there is a mechanism which somehow keeps the established viral genome in a latent state unless proper conditions are applied. Once these conditions are fulfilled, R=1 and reactivation is effective (r=1) after a delay t_r.

Now, we are in a position to propose equations for V and A.
$V = \bar{a}\ (i_v + i_a + r)$ means that provided the level of immunity against the virus is below a threshold ($\bar{a}$), virus multiplication can take place following infection (i) or reactivation (r). Infection with virulent (i_v) or with attenuated (i_a) virus both result in virus development (V), but with different "on" and "off" delays (table 2).
$A = v + v_i + r$ means that the development of specific immunity against the virus is induced by a successful multiplication of the virus (v) or by the massive injection of inactivated virus or by reactivation (r).
We thus reach the following provisional description (figure 2):

$$V = \bar{a}\ (i_v + i_a + r)$$
$$R = gd$$
$$A = v + v_i + r$$
$$G = v + g$$
$$M = a + m$$

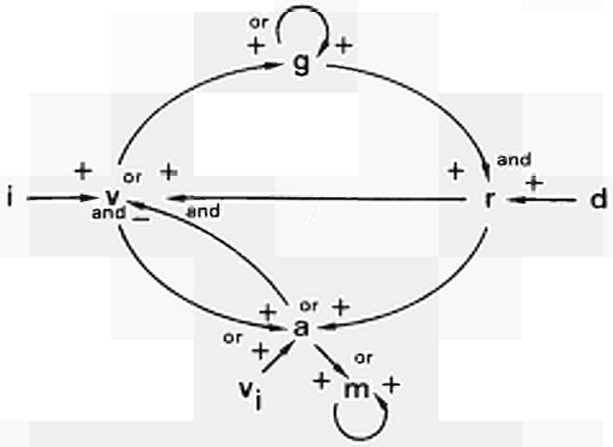

Fig. 2 Logical scheme of the BHV 1 latent infection.

Note that i_v, i_a, v_i and d are input variables: whether or not infection, injection of inactivated virus or an appropriate reactivation stimulus take place does not depend on the internal state of the system but rather on a deliberate decision of the experimenter or on casual external circumstances. The other variables (v, r, a, g, m) are internal variables whose value depends on earlier values of each other. An essential point is that we do not try to mention in the equation of a function all the variables which influence the value of this function, but rather those variables which (rightly or wrongly) are considered to influence directly the function. For instance, variable v influences its own development, but only indirectly. This is why v is not seen in the equation of V. But v directly influences the development of immunity (and it is thus present in the equation of A) and a in turn influences the multiplication of the virus (and it is thus present in the equation of V). Thus v figures in the equation of A and a figures in the equation of V; this describes the indirect effect of v on its own later development.

The values of the time delays are expressed in "units of time delay", which correspond roughly to days. They represent the time required, under experimental conditions, to switch on or off the variables when the values of the corresponding function has changed. It is assumed, for example, that the level of neutralising antibodies and of cell-mediated immunity has reached a protective threshold ten days after primary infection. We therefore chose the value 10 for t_{a1}. Other values of time delays have been arbitrarily chosen: for instance, t_{τ} value cannot be deduced from experi-

tal data and was therefore considered as equal to 1 (table 2).

TABLE 2 Time delays

		$i_v = 1$	$i_a = 1$		
m = 0	t_{a1}:	10	10	$t_g = 0$	$t_v = 1$
	$t\bar{}_{a1}$:	56	28	$t\bar{}_g$: no value	$t\bar{}_v = 12$
m = 1	t_{a2}:	7	7	$t_m = 0$	$t_r = 1$
	$t\bar{}_{a2}$:	180	90	$t\bar{}_m$: no value	$t\bar{}_r = 1$

The delay values are expressed in units of time delay;
one unit corresponds roughly to one day;
t_x : delay of appearance of the variable x;
$t\bar{}_x$: delay of disappearance of the variable x.

From our set of logical equations, one can derive a state table (table 3) which provides the values of the functions for each combination of values of the variables. Usually, each of the 2^n combinations of values of the n internal variables occupies a row. In the present case, however, instead of writing a big table with 32 rows (corresponding to the combinations of values of the 5 internal variables), we give two five-variable-sub-tables, one for m=0, one for m=1. This is convenient because in practice one begins in the sub-table (m=0), and as soon as there has been a primary response, one shifts to (and remains in) the sub-table (m=1).

TABLE 3 Complete state table

m = 0	VRAGM				m = 1	VRAGM			
vragm	0	v_i	i	d	vragm	0	v_i	i	d
00000	00000	00100	10000	00000	00001	00001	00101	10001	00001
00010	00010	00110	10010	01010	00011	00011	00111	10011	01011
00110	00011	00111	00011	01011	00111	00011	00111	00011	01011
00100	00001	00101	00001	00001	00101	00001	00101	00001	00001
01100	00101	00101	00101	00101	01101	00101	00101	00101	00101
01110	00111	00111	00111	01111	01111	00111	00111	00111	01111
01010	10110	10110	10110	11110	01011	10111	10111	10111	11111
01000	10100	10100	10100	10100	01001	10101	10101	10101	10101
11000	10110	10110	10110	10110	11001	10111	10111	10111	10111
11010	10110	10110	10110	11110	11011	10111	10111	10111	11111
11110	00111	00111	00111	01111	11111	00111	00111	00111	01111
11100	00111	00111	00111	00111	11101	00111	00111	00111	00111
10100	00111	00111	00111	00111	10101	00111	00111	00111	00111
10110	00111	00111	00111	01111	10111	00111	00111	00111	01111
10010	00110	00110	10110	01110	10011	00111	00111	10111	01111
10000	00110	00110	10110	00110	10001	00111	00111	10111	00111

Each table (or sub-table) comprises columns corresponding to the various possible treatments (input variables). Here, we have considered columns corresponding to: no present treatment (o); injection of inactivated virus (v_i); infection with a live virus (i); treatment with dexamethasone or a similar reactivating stimulus (d). We have not considered columns corresponding to simultaneous double or multiple treatments; however, one can perfectly go, for example, from column 0 to column i (infection), back to column 0 and then to column d (stimulus of reactivation).

Note that each logical state of the system can be described by a logical vector listing the values of the variables and a second logical vector listing the corresponding values of the functions. Consider, for instance, a virgin animal which has just been vaccinated with inactivated virus. In this situation, the state of variables is 00000 (first line of table 3, left column), but the state of functions is 00100 (same line, column v_i), indicating that function A (the development of specific immunity against the virus) is on. One can describe this state of the system by giving the vector of variables followed by the vector of functions: 00000/00100.

However, it is more compact and more convenient to write 00$\bar{0}$00, in which the dash over the value of the third variable indicates that there is an order to change its value; indeed, specific immunity is not yet present, but it is being developed (as a result of vaccination). Note that a state without any dash is a stable state, as there is no order to change the value of any variable. See, for instance, state 00011 in the right part of table 3, second row, column 0: this describes a carrier state in which there is neither mature virus nor a state of reactivation nor specific immunity, but the viral genome and a memory of an earlier immunisation are present.

It has been found convenient to re-write, after a state table, a "compact" state table in which each state is described by the logical vector of the variables, completed by the dashes which indicate which variables are asked to commute. In the present case, this gives table 4.

DYNAMICAL ANALYSIS

Let us start from 00000, a stable state of column 0 (table 4) which represents a "virgin" animal free of BHV 1 infection and of any previous contact with BHV 1 antigen. Infection is seen in our formalism as a shift from column 0 to column i. Strictly speaking, the system should remain in

TABLE 4 Compact state table

m = 0				m = 1			
0	v_I	I	d	0	v_I	I	d
00000	00$\bar{0}$00	$\bar{0}$0000	[$\bar{0}\bar{0}\bar{0}\bar{0}\bar{0}$]	00001	00$\bar{0}$01	$\bar{0}$0001	[$\bar{0}\bar{0}\bar{0}\bar{0}$1]
00010	00$\bar{0}$10	$\bar{0}$0010	00$\bar{0}$10	00011	00$\bar{0}$11	$\bar{0}$0011	00$\bar{0}$11
00$\bar{1}$1$\bar{0}$	0011$\bar{0}$	00$\bar{1}$1$\bar{0}$	00$\bar{1}$1$\bar{0}$	00$\bar{1}$11	[00$\bar{1}\bar{1}\bar{1}$1]	00$\bar{1}$11	00$\bar{1}$11
00$\bar{1}$0$\bar{0}$	0010$\bar{0}$	00$\bar{1}$0$\bar{0}$	00$\bar{1}$0$\bar{0}$	00$\bar{1}$01	[00$\bar{1}$0$\bar{1}$1]	00$\bar{1}$01	00$\bar{1}$01
0$\bar{1}$10$\bar{0}$	0$\bar{1}$10$\bar{0}$	0$\bar{1}$10$\bar{0}$	0$\bar{1}$10$\bar{0}$	0$\bar{1}$101	0$\bar{1}$101	0$\bar{1}$101	0$\bar{1}$101
0$\bar{1}$11$\bar{0}$	0$\bar{1}$11$\bar{0}$	0$\bar{1}$11$\bar{0}$	01110	0$\bar{1}$111	0$\bar{1}$111	0$\bar{1}$111	[0$\bar{1}\bar{1}\bar{1}\bar{1}$]
$\bar{0}$1$\bar{0}$10	$\bar{0}$1$\bar{0}$10	$\bar{0}$1$\bar{0}$10	$\bar{0}$10$\bar{1}$0	$\bar{0}$1$\bar{0}$11	$\bar{0}$1$\bar{0}$11	$\bar{0}$1$\bar{0}$11	$\bar{0}$10$\bar{1}$1
$\bar{0}$1$\bar{0}$00	$\bar{0}$1$\bar{0}$00	$\bar{0}$1$\bar{0}$00	$\bar{0}$1$\bar{0}$00	$\bar{0}$1$\bar{0}$01	$\bar{0}$1$\bar{0}$01	$\bar{0}$1$\bar{0}$01	$\bar{0}$1$\bar{0}$01
1$\bar{1}\bar{0}\bar{0}$0	1$\bar{1}\bar{0}\bar{0}$0	1$\bar{1}\bar{0}\bar{0}$0	1$\bar{1}\bar{0}\bar{0}$0	1$\bar{1}\bar{0}\bar{0}$1	1$\bar{1}\bar{0}\bar{0}$1	1$\bar{1}\bar{0}\bar{0}$1	1$\bar{1}\bar{0}\bar{0}$1
1$\bar{1}$10	1$\bar{1}$10	1$\bar{1}$10	11$\bar{0}$10	1$\bar{1}$11	1$\bar{1}$11	1$\bar{1}$11	11$\bar{0}$11
$\bar{1}\bar{1}$11$\bar{0}$	$\bar{1}\bar{1}$11$\bar{0}$	$\bar{1}\bar{1}$11$\bar{0}$	$\bar{1}$1110	$\bar{1}\bar{1}$111	$\bar{1}\bar{1}$111	$\bar{1}\bar{1}$111	$\bar{1}$1111
$\bar{1}\bar{1}$10$\bar{0}$	$\bar{1}\bar{1}$10$\bar{0}$	$\bar{1}\bar{1}$10$\bar{0}$	$\bar{1}\bar{1}$10$\bar{0}$	$\bar{1}\bar{1}$101	$\bar{1}\bar{1}$101	$\bar{1}\bar{1}$101	$\bar{1}\bar{1}$101
$\bar{1}$0$\bar{1}$0$\bar{0}$	$\bar{1}$0$\bar{1}$0$\bar{0}$	$\bar{1}$0$\bar{1}$0$\bar{0}$	$\bar{1}$0$\bar{1}$0$\bar{0}$	$\bar{1}$0$\bar{1}$01	$\bar{1}$0$\bar{1}$01	$\bar{1}$0$\bar{1}$01	$\bar{1}$0$\bar{1}$01
$\bar{1}$0$\bar{1}$1$\bar{0}$	$\bar{1}$0$\bar{1}$1$\bar{0}$	$\bar{1}$0$\bar{1}$1$\bar{0}$	$\bar{1}\bar{0}$11$\bar{0}$	$\bar{1}$0$\bar{1}$11	$\bar{1}$0$\bar{1}$11	$\bar{1}$0$\bar{1}$11	$\bar{1}\bar{0}$111
$\bar{1}$00$\bar{1}$0	$\bar{1}$00$\bar{1}$0	100$\bar{1}$0	$\bar{1}\bar{0}$0$\bar{1}$0	$\bar{1}$00$\bar{1}$1	$\bar{1}$00$\bar{1}$1	100$\bar{1}$1	$\bar{1}\bar{0}$0$\bar{1}$1
$\bar{1}$0$\bar{0}\bar{0}$0	$\bar{1}$0$\bar{0}\bar{0}$0	10$\bar{0}\bar{0}$0	$\bar{1}$0$\bar{0}\bar{0}$0	$\bar{1}$0$\bar{0}\bar{0}$1	$\bar{1}$0$\bar{0}\bar{0}$1	10$\bar{0}\bar{0}$1	$\bar{1}$0$\bar{0}\bar{0}$1

this column just for the actual duration of the exposition to exogenous virus. This could be decribed by ascribing to the process a specific time delay or by introducing an additional variable to memorize the exposition to virus. In practice, it appeared more convenient and not too distorting to keep the system in a column other than column 0 just for the time required to switch one variable; afterwards, the system goes back to the initial column (figure 3).

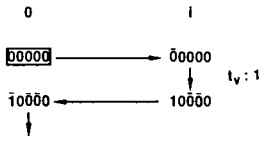

Fig. 3 Pathway followed after infection of a virgin animal

The system is now in state $\bar{1}0\bar{0}\bar{0}0$, in which three variables are asked to change their value; the change that will actually take place depends on the values of the time delays (table 2). We assume that once the animal is successfully infected, the time required to establish the viral genome is short and at any rate shorter than the two other time delays, which we estimate to 10 units of delay (UD) for the development of an efficient

specific immunity and at about 12 UD for the disappearance of mature viral particles. With these values of the delays, the pathway is as described in figure 4 with as successive steps: permanent establishment of the viral genomes (g); establishment of specific immunity (a). The state is now $\bar{1}011\bar{0}$; as soon as specific immunity is developed, m (which serves to remind that there has been a primary response) takes the value 1 and we reach state $\bar{1}0111$ (column 0 in the right part of table 4, which corresponds to the situation m=1). From now, there is only one possible evolution: $\bar{1}0111 \rightarrow 00\bar{1}11 \rightarrow$ [00011] , in which the virions and immunity disappear after some days and some weeks, respectively. The animal is now in a stable state [00011] in which the viral genome is still present (latent state) and there is a memory of a primary specific immune response.

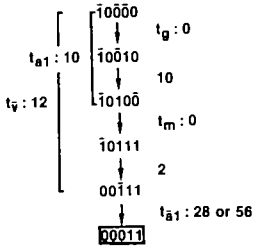

Fig. 4 Pathway followed after infection of a virgin animal (following)

Starting from state [00011] , various stimuli (e.g. treatment with dexamethasone) can lead to column d, resulting in reactivation (figure 5).

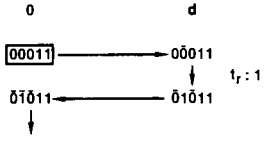

Fig. 5 Application of stimulus of reactivation

The system now goes back to column 0: the stimulus itself has disappeared, but the organism keeps for a while (1 UD) a memory of the stimulus, which is symbolized by function R (reactivation), but which mechanism is still unknow. Virus is produced as early as 1 UD after the application of the stimulus, followed by the development of a specific immune response; here, one

is dealing with a secondary response (m=1), therefore the "on" delay is much shorter (t_{a2}=7) than the first time and the "off" delay much longer ($t_{\bar{a}2}$= 90 or 180) (table 2). The sequence is shown in figure 6:

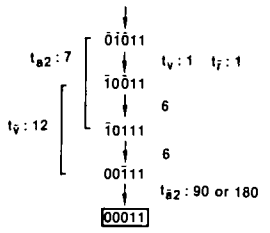

Fig. 6 Pathway followed after reactivation

Another possibility would consist in promoting reactivation very soon after primary infection, before a specific immune response has developed. According to our scheme, the evolution would be (figure 7):

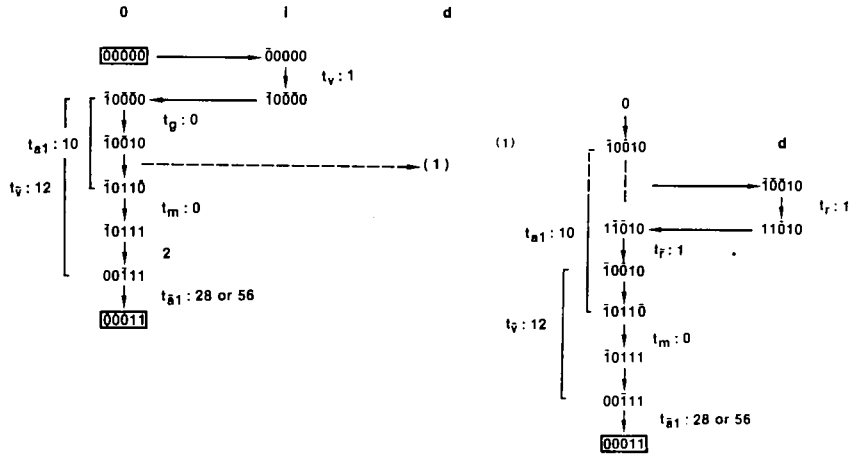

Fig. 7 Pathway followed after reactivation induced very soon after primary infection

A characteristic feature of this scenario is that reactivation provides a counterorder to the order to stop virus production and the new order to stop this production takes place only after the state of reactivation has disappeared. Here we would expect virus to persist 14 to 22 UD in contrast with the normal period of 12 UD. Although there are no available experiments in which a dexamethasone treatment has been applied soon after primary infection, partly similar experiments have been performed in which, 10 days after primary infection, animals have been infested with *Dictyocaulus viviparus* , which also induces reactivation (Msolla et al., 1983). Virions are indeed found up to 3 weeks after primary infection, precisely until day 13 after primary infection and also at day 20 (Thiry et al., 1985b).

The injection of inactivated virus provokes the immunization of the animal, as indicated in the logical equation: $A = v + v_i + r$. This procedure can be used to impede the reexcretion of infectious particles by cattle in which a latent BHV 1 is reactivated. The pathway followed is detailed in figure 8:

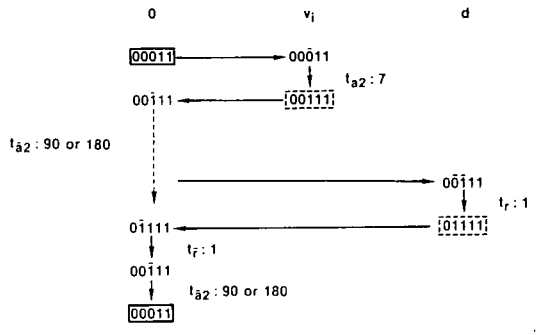

Fig. 8 Prevention of BHV 1 reexcretion by injection of inactivated antigens.

It can be shown that no re-excretion occurs when the reactivation stimulus is applied at least 7 UD after the injection of inactivated antigens. Nevertheless, the effect of reactivation on specific immunity is not modified and the order of disappearance of variable a is lost during the period when r=1. This situation was precisely observed in latently infected cattle in which a booster effect was provoked by delayed

hypersensitivity test (DHT) (Thiry et al., 1983a). DHT antigen is inactivated BHV 1 virus and, when dht is applied on sensitized animals, it induces a booster response (Brochier et al., 1984): this mimics the situation provoked by $v_i=1$, but only in the case of a booster response, because DHT does not induce any primary immune response (at least the humoral one) in virgin animals (Thiry et al., 1983b).

CONCLUSIONS

The formalized description gives a coherent, even if simplified, picture of the sequences of events which follow BHV 1 infection, reactivation or vaccination with inactivated antigens. When examining the cases described in the dynamical analysis, the description shows that it is in accordance with experimental observations. Therefore, the logical description must be essentially considered as a tool in research work and in the analysis of epizootiological situations. It makes possible a schematic representation of an experimental schedule and helps to choose the most appropriate schedule. It gives a prevision of the evolution of the interactions between BHV 1 and the infected cattle, in a given epizootiological situation.

This logical description has to be refined in the light of new experimental data: a special attention mut be given to a more detailed description of the immunological parameters (e.g. dissociation between humoral and cell-mediated immunity) and to the mechanism of reactivation (to assign experimental values to the time delays t_r and $t_{\bar{r}}$). Nevertheless, even in its present state, this logical description has already allowed us to use a more systematic approach in the analysis of BHV 1 latent infection, to propose solutions to some unanswered questions and to suggest new experiments.

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COMMON SEROLOGICAL DIAGNOSTIC METHODS FOR BHV 1 INFECTIONS IN EUROPE

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ABSTRACT

The most widely used test is the serum neutralization test, which is considered specific, sensitive and reproducible. Its drawback is the rather long time it takes to obtain the results. The next most frequently used technique is the ELISA, which takes only hours. Its disadvantage is a lower specificity which leads to a number of false positive results. Fast is also the radioimmunoassay, which can be conducted even with contaminated sera, but requires highly purified antigen preparations and the use of radioactive material. Other tests less frequently employed are the immunofluorescence, a passive hemagglutination and immunodiffusion test, a counter-immunoelectro-osmophoresis and a micro complement fixation test.

INTRODUCTION

BHV 1 infections were until the middle of this century confined to the genital tracts of male and females, where they caused the disease named coital exanthema. But little attention was given to the causative agent, which had been known to be a virus since 1928 (Reisinger and Reimann). When the etiological agent of the then new disease Infectious Bovine Rhinotracheitis (IBR) was discovered in the USA (Madin et al., 1956), the first serological method introduced was the serum neutralization test (McKercher et al., 1959a). This reliable test was the basis for the surprising finding that the etiological agents of coital exanthema and IBR were serologically identical (Gillespie et al., 1959; McKercher et al., 1959b). Since then serologically identical agents have been recovered from cases of encephalitis (French, 1962), conjunctivitis (Abinanti and Plummer, 1961; McKercher et al., 1959a), abortion (McKercher and Wada, 1964), mastitis (Greig

and Bannister, 1969; Straub and Kielwein, 1966) mostly overseas, but in recent years also in Europe (for review s. Straub, 1978, and Straub et al., 1982). These virus recoveries led to an enormous stimulus to establish laboratory methods for the serological diagnosis.

THE SERUM NEUTRALIZATION TEST (SNT)

The original components, serum and virus suspension, are still in use in 1984. Trials to improve test results by adding complement have not fulfilled the expectations. The test is conducted in manifold variations which have in common only the use of serial serum dilutions and a constant virus suspension for the determination of the antibody titres, or serial dilutions of virus suspensions and a constant serum dilution for the identification of viral isolates. The other parameters vary from laboratory to laboratory. Many different cells are used and in a comparative test conducted in EC laboratories these included fetal bovine kidney, bovine kidney, AUBEK, MDBK and GBK cells (Lorenz and Straub, unpublished data). The techniques employed included a tube test, a micro test, and in another trial the plaque reduction test. Other variables were the $TCID_{50}$s, growth and maintenance media, virus strains, temperature and time of incubation and finally the methods of estimation. Despite these variations there is general agreement that the test is (1) sensitive (2) reproducible (3) highly specific (4) easy to conduct (5) reacting with all classes of immunoglobulins (Matthaeus and Straub, 1977) but unable to differentiate between the causative agents of the various diseases which led to the antibody formation.

THE ELISA

In many antibody determinations this test has proven its superiority over other laboratory methods, especially the SNT as far as expenditures are concerned. In EC countries there are two commercial sets available which enable laboratories to conduct mass examinations in a relatively short period of

time. In comparative trials its sensitivity is highly rated, but in the doubtful range we could not confirm this advantage: on the contrary, its rather low specificity led to a number of false positive results. The reason for this apparent discrepancy is based on the components of the reacting agents. If an animal has suffered from a BHV 1 diseaseimmunoglobulins of the classes M, G and A are formed but only those of class IgG can be detected in the ELISA. If on the other hand an animal is only vaccinated parenterally then the ELISA and SNT titres should - provided the IgM class antibodies have already largely disappeared - give comparable results. In Table 1 an example is presented (Straub, 1984b).

TABLE 1 A comparison between results obtained in the ELISA and SNT.

Mode of Vaccination	Animals no.	Titres in serum determinded by ELISA[1)]	SNT[2)]
2 x parenteral	174	10 240	2.85
	182	7 680	2.55
	412	480	2.10
1 x parenteral 1 x local	179	320	1.80
	185	160	1.95
	184	10	1.50
field virus infection	158M	240	2.85

1) - reciprocal values
2) - in $-\log_{10}$

THE PASSIVE HAEMAGGLUTINATION TEST

This procedure was first described by Vengris and Maré

(1971) and recently by Dannacher et al. (1979). For references see Straub (1978). When we tried the first few tests described we could not confirm their efficiency and when the latest one was tried by the senior author in the comparative EC test it did not work either. At present there is obviously no passive haemagglutination test which can give reliable, reproducible results.

THE IMMUNODIFFUSION TEST (IDT)

Although a precipitation test has been described as early as 1967 (Estela) there is no recent report available that makes use of this test. There are, however, two publications in preparation (Spieck and Straub), were the IDT has been used. In our own work the IDT was compared with the results obtained by the SNT and the technique used was the same as for the serological diagnosis of enzootic bovine leukosis (EBL) (Straub 1984a). The test appears as highly specific and sensitive enough to detect antibody titres following an acute infection.

THE RADIOIMMUNOASSAY (RIA)

This test was found to be 320- to 150 000-fold more sensitive than the SNT in a comparative trial with swine herpes virus (Aujeszky) (Döller and Jakubik, 1980). Advantages are its rapidity and the possibility of using contaminated sera, but at present the necessity to use radioactive material appears to be an obstacle as is the requirement to have highly purified antigen available.

THE IMMUNOFLUORESCENCE TEST (IFT)

The IFT is most frequently used for the detection of antigen (Wellemans and Leunen, 1973), but it can also be used for the detection of antibodies as described by Bergmann and Hahnefeld (1967). Since it requires higher expenditures and skilful handling it has never been widely used.

OTHER METHODS

There are some other methods described which, however, have never been widely used. These include counter-immuno-electro-osmophoresis (Aguilar-Setién al. (1980), and a micro complement fixation test (Karadzhov and Khristova, 1980).

DISCUSSION

All the tests described can in the hands of experts lead to comparable results. The most widely used test appears to be the SNT. Its main drawback is certainly the period of time necessary to obtain the result, which as far as the ELISA is concerned is a major advantage. The higher sensitivity of the ELISA versa the SNT has led to the introduction of the ELISA for testing milk and even bulk milk samples, as now commonly used in Switzerland (de Meuron, 1982; Stuker et al., 1980). On the other hand the lower specificity of the ELISA results in a number of false positives. The use of monoclonal antibodies in the ELISA has improved the quality of the EBL diagnosis (Mammerickx et al., 1984). Whether the same will be true for BHV 1 diagnosis remains to be determined. It would certainly improve the diagnostic horizon if it would be possible to establish an IgA ELISA. On the other hand the load of laboratory work already imposed on diagnostic centres may be lightened when the diagnosis can be based on a skin test. This is presently being tested on a large scale using a commercially produced antigen.

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ON CONTROL AND ERADICATION OF INFECTIOUS BOVINE RHINOTRACHEITIS

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ABSTRACT

Control of infectious bovine rhinotracheitis (IBR) can still not be achieved by vaccination. It is therefore important to prevent introduction of IBR into uninfected areas and uninfected herds and to eradicate in infected herds. Relevant epidemiological features of the disease and principles of control and eradication are summarized. It is emphasized that tests used to disclose infected herds and infected animals and to secure freedom from infection should be both very sensitive and specific. The sensitive and specific tests used in Denmark are briefly described.

INTRODUCTION

It still seems to be generally accepted that it is impossible to control infectious bovine rhinotracheitis (IBR) and Aujeszky's disease - two herpes virus infections - by use of vaccination. The best solution would therefore be to prevent introduction of such infections into uninfected areas and to eradicate in infected areas. This would presuppose exact knowledge of the epidemiology and the use of reliable tests to disclose infection or freedom from infection in herds as well as in individual animals.

In Denmark, IBR has been subject to control in artificial insemination centres and progeny testing stations for many years (cf. Bitsch, 1984), but in February 1984 the farmers' organizations initiated a nation-wide volontary eradication supported by legislation. A control program concerning Aujeszky's disease was introduced some years ago, and the final goal is now within sight (Andersen et al., 1984).

In the following will be mentioned the most important epidemiological features of IBR with respect to infection control as well as aspects of serological control testing, including requirements concerning serological tests.

RELEVANT EPIDEMIOLOGICAL FEATURES

The intermittent release of virus from infected animals is of utmost importance. The amounts of virus shed during periods of recurrent excretion are generally much lower than during the acute phase of infection (Bitsch 1984). With regard to control and eradication, uninfected animals should therefore not be allowed to have contact with earlier infected ones. Nevertheless, on an infected premises eradication should have a reasonable chance of success because of the low amounts of virus re-excreted, even when infected and uninfected animals are on the same farm, but separated from each other.

It should be noted that in areas where artificial insemination is used, control is likely to be successful only when the semen used originates from centres free of IBR virus infection.

Regarding eradication of Aujeszky's disease it is essential to eradicate region-wise (Bitsch and Andersen, 1982), but as with IBR no evidence of an airborne herd-to-herd transmission has been seen, eradication can be carried out in individual herds irrespective of a possible presence of the infection in neighbouring herds. But co-ordinated, area-wise control will reduce the risk of a spreading between herds, for example by man.

CONTROL AND ERADICATION

In uninfected areas it is essential to establish surveillance testings of herds and in infected areas to restrict movement of animals, which should be allowed only after serological testing of the individual animals or the herds of origin.

Test of bulk milk samples at regular intervals as performed in Switzerland (Kihm and Bommeli, 1981) and now in Denmark will serve not only a surveillance purpose but also to disclose the infected herds, which is necessary in the initial phase of the eradication. Control testing of herds of beef cattle will have to be based on examination of blood samples.

After a serological examination of all animals in infected herds, eradication can in principle be done in two ways, i.e., either by replacing the whole herd by animals from uninfected

herds, or by separating infected animals from uninfected ones and slaughtering of the infected animals when found appropriate.

As it may be costly to replace a whole infected herd, it may be advantageous in such cases to rear instead the new calves isolated from the rest of the animals in order to establish a new herd on basis of such animals.

If only a few animals are infected, they should of course be removed as soon as possible.

If a somewhat higher proportion is infected, the infected animals should be kept apart from uninfected, preferably in separate houses. In the course of a very few years such herds should have a very good chance to be free from infected animals.

IBR ANTIBODY TESTS

Virus-neutralizing antibody (VNA) tests and ELISA tests are most commonly used for demonstration of antibody to IBR virus. It is essential that the tests used in infection control are enough sensitive and specific.

Unfortunately, VNA test modifications used at many laboratories have not demonstrated fulfilment of these requirements. The Danish P37/24 modification, however, being 16 times more sensitive than a conventional test, has proved to be sensitive enough for practical use, and its specificity is still as good o.999 when serum samples are tetsted undiluted (Bitsch, 1978).

From examinations with ELISA tests based on enzyme-labelled anti-bovine immunoglobulin it was concluded that these tests were not as sensitive as the P37/24 VNA test. However, in work on Aujeszky's disease a blocking, direct ELISA test was developed, which fulfilled the above requirements (Bitsch and Meyling, unpublished; partly on basis of results obtained by Sørensen, unpublished), and an IBR blocking, direct ELISA test was elaborated thereafter (Bitsch, Meyling and Abraham, unpublished). This ELISA test appeared to be just as sensitive and specific as the P37/24 VNA test, but was much easier to perform, faster and extremely inexpensive. It has been used routinely now for more than one year with excellent results.

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Goat herpesvirus infections: a survey on specific antibodies in different countries.

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Abstract

Goat herpesvirus isolates are reported from Norway and New Zealand. Five different isolates were neutralized and belong to one serogroup. An antibody survey with goat sera from European and Asian countries shows that goat herpesvirus infections seem to be widely spread and latent virus carriers must be present in these countries.

INTRODUCTION

Herpesviruses from goats have been isolated in California (Saito et al., 1974) and Switzerland (Mettler et al,. 1979). The viruses isolated from severely diseased goat kids have been characterized and shown to be closely related to bovine herpesvirus type 1 (Engels et al., 1983). In the past it was shwon that goats can be infected by BHV-1 exhibiting weak symptoms (McKercher et al., 1959).The goat herpesvirus has been grouped as BHV-6 (Ludwig, 1983). Since in a variety of countries in the world the goat is of economic importance we searched for other BHV-6 strains and specific antibodies. The data presented in this report show that goat herpesviruses are spread worldwide.

MATERIALS AND METHODS

The E/CH and McK strains were used as references. Other strains obtained are listed in Table 1. The viruses (including IBR- and IPV reference virus strains), were grown in GBK cells follwing standard conditions. Neutralization tests were performed using the plaque reduction assay (Pauli and Ludwig, 1977) in order to differentiate BHV-6 isolates from BHV-1. A plaque immuno assay (Pauli et al., 1985) served to screen sera for the presence of anti-E/CH antibodies. Goat sera and a limited amount of information on disease problems in goats were received from co-workers in those countries listed in Table 1 and 3.

RESULTS AND DISCUSSION

Several isolates obtained from adult goats with infections of the genital tract or even isolated from lymphocytes of healthy animals (Table 1) have been compared in neutralization tests with reference goat herpesvirus and BHV-1. As shown in Table 2, these isolates could be classified as BHV-6 according to serological criteria. Hyperimmune serum as well as goat convalescent sera clearly show that no serological divergency exists between the goat herpesviruses.

Isolate	Country	Site of isolate	Reference	Passage
1- McK/US	California (1974)	Organ pool	Saito	10
2- E/CH	Switzerland (1979)	Organ pool	Mettler	10
3- CHV-9251-84	New Zealand (1984)		Horner	2
4- CHV-44/NOR	Norway (1984)	Goat prepuce	Hyllseth	1
5- CHV- 80/NOR	Norway (1984)	Female goat leukocytes	Hyllseth	1

Table 1: Goat herpesvirus (BHV-6) isolates identified by serology

This group of viruses can readily be differentiated from IBR/IPV viruses (BHV-1) by neutralization . Goat herpesvirus specific goat antisera neutralize BHV-1 considerably less than the homologous goat viruses (Table 2).

	Titre using antiserum against				
	E/CH+ (goat)	IBR (cattle)	CHV-5YR. (goat)	CHV-44/NOR. (goat)	CHV-6R. (goat)
Virus					
1- E/CH	1:1024	< 1:8	1:256	1:128	1:256
2- McK/US	1:1024	1:128	1:256	1:128	1:256
3- CHV-44/NOR	1:1024	1:64	1:256	1:128	1:256
4- CHV-80/NOR	1:1024	1:64	1:256	1:128	1:256
5- CHV-9521-84	1:1024	1:64	1:256	1:128	1:256
6- IBR	<1:8	1:512	1:8	1:4	<1:8
7- IPV	<1:8	1:128	<1:8	<1:8	<1:8

Table 2: Cross-neutralization of BHV-6 isolates originating from different countries using cattle and goat antisera.
Titres are expressed as the serum dilution neutralizing 80% of the plaques.
+ Serum after experimental infection.

Goat sera from a variety of countries were screened for the presence of antibodies (Table 3). It could be shown that - although viruses were isolated in some of these countries and the sera derived from healthy animals - BHV-6 is latently present in animals of these countries. Some of the animals have relatively high neutralizing titres (up to 1:250) indicating that recurrent infections must have occurred.

COUNTRY	NEUTRALIZATION TEST NEUTRALIZING ANTIBODIES AGAINST		ELISA TEST+
	BHV-1	BHV-6	
1- BANGLADESH	N.T	0/5++	0/5
2- GERMANY	0/100	0/100	0/100
3- GREAT BRITAIN	N.T	0/19	4/19
4- GREECE	4/126	126/128	135/224
5- ISRAEL	N.T.	0/6	6/24
6- ITALY	2/2	3/3	3/112
7- NORTHERN IRELAND	N.T	11/101	12/101
8- NORWAY	1/4	4/134	4/134
9- SPAIN	1/14	5/92	9/33
10- SYRIA	N.T.	3/13	N.T.
11- SWITZERLAND	N.T.	0/2000	0/2000
12- TURKEY	N.T	6/7	11/20

Table 3 Serological survey on BHV-6 antibodies
+ Sera tested on BHV-6 (E/CH) infected GBK cells.
++ No. sera positive/sera tested.
N.T. not tested

A comparison of the neutralization and the ELISA titres from sera of seven countries where positive animals had been found is presented in Figure 1. The data indicate that ELISA titres were more than 10 fold higher than neutralization titres. All the neutralizing sera were clearly positive in the ELISA, however, vice versa approx. 10% of the sera showed ELISA reactions and had neutralization titres lower than 1:2. Positivity of a goat serum was in all cases based on neutralization with a serum dilution of at least 1:2.

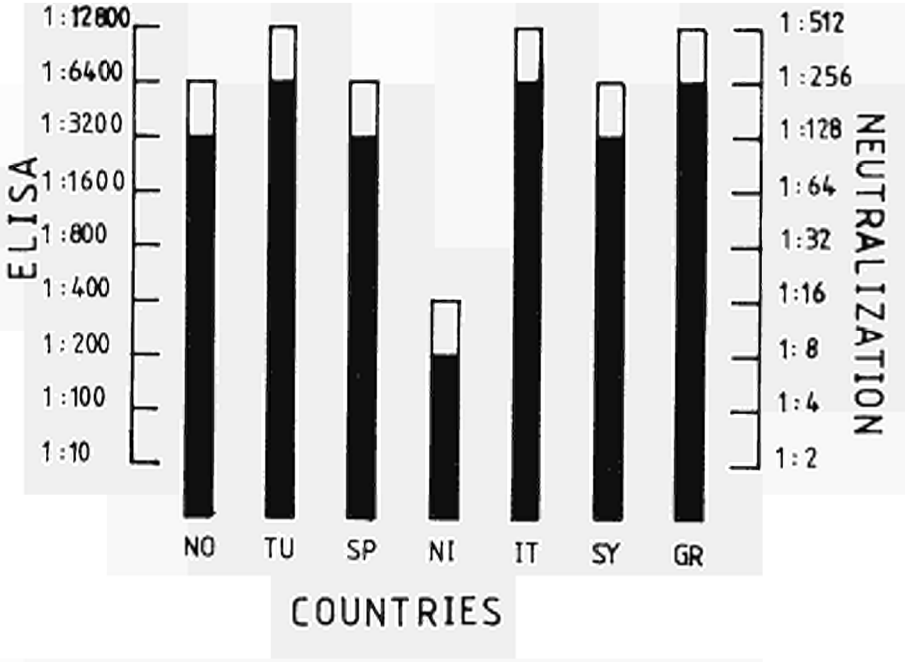

Fig. 1: Comparison of neutralization and ELISA titres of goat sera obtained from European and Asian countries. The Swiss strain (E/CH) served as reference.
NO = Norway; TU = Turkey; SP=Spain; NI = Northern Ireland; IT = Italy; GR = Greece
ELISA titre
Neutralization titre

Screening of several hundred goat sera has brought valuable information on the spread of goat herpesviruses in several countries. The situation concerning this virus which is indigenious to the goat, seems to be reminiscent of BHV-1 infections in cattle (Straub, 1978). From the data presented it may be predicted that BHV-6 is spread in many countries and under immuno compromised conditions might be hazardous for its host leading to severe economic losses.

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STUDIES ON BOVID HERPESVIRUS 2: PATHOGENESIS OF THE INFECTION AND THE IMMUNOLOGIC RELATIONSHIP WITH HERPES SIMPLEX VIRUS

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ABSTRACT

The results of studies on the pathogenesis of bovid herpesvirus 2 (BHV2) and the possibility that the virus remains as a latent infection in cattle, were reviewed. From those studies it seems reasonable to conclude that the pathogenesis of BHV2 infection in cattle might depend on the route of exposure to the virus, i.e., intravenous inoculation produces a systemic condition, whereas intradermal or intranasal exposure are followed by a localized disease. The study on latency of the virus supports the possibility that BHV2 induces a latent infection in cattle. This study suggested also that the skin, and probably the nervous system, are the host sites where the virus persists in the latent form.

From the immunologic relationship study it was found that Herpes simplex virus (HSV) partially protected calves against experimentally induced BHV2 infection. The most significant serologic finding was that the HSV-preimmunized calves produced antibody to BHV2 challenge virus at earlier time than did the control calves.

INTRODUCTION

Bovid herpesvirus 2 (BHV2) is the etiologic agent of a trasmissible disease of cattle and buffalo, characterized by the appearance of nodules, vesicles, ulcers and scabs on the skin and oral mucosa. Since a strain of BHV2 was isolated in Italy (Castrucci et al., 1972), some aspects on the pathogenesis of the infection have been investigated and also a program on the immunologic relationship between this virus and the Herpes simplex virus (HSV) was planned. The researches on the pathogenesis included the behavior of the virus in the target organs of the natural host and the latency of the infection in experimentally infected cattle (Castrucci et al., 1977; 1978; 1979; 1982). The study on the immunologic relationship between BHV2 and HSV, was undertaken with the purpose of verifying whether, because

of the common antigen, a viral envelope glycosylated protein (Norrild et al., 1978), shared by the two viruses (Ludwig, 1983), the HSV could protect calves against challenge with BHV2 (Castrucci et al., 1984). The present report summarizes the findings made.

MATERIALS AND METHODS

BHV2: the Italian strain 69/1LO (Castrucci et al., 1972) at its 2nd passage level in bovine embryo kidney (BEK) cell cultures was used. The titer was of $10^{6.50}$ $TCID_{50}$/0.05 ml.

HSV: strain 5007 of HSV1 and strain 2248 of HSV2 were used at the 3rd passage level in BHK-21 line cells. The two strains were kindly supplied by Dr. G.R.B. Skinner, University of Birmingham, G.B., and the titers were $10^{8.50}$ or $10^{7.50}$/ml, respectively, for HSV1 and HSV2. For immunizing the calves the HSV1 and HSV2 antigens were inactivated by a 0.5% solution of Triton 100X.

The behavior of BHV2 in the target organs of calves was studied in 38 approximately 2 month-old Simmenthal calves, devoid of antibody to the virus, of which, 18 were infected intravenously (i.v.), 10 intradermally (i.d.) and 10 intranasally (i.n.). In each group, calves were killed at predetermined intervals, each animal was necropsied and samples (Table 1) were obtained for histopathologic examinations and for culture of virus.

The study on latency was conducted in 7 Simmenthal calves, approximately 6 months of age, which had recovered from experimental infection induced i.v. (3 calves) or i.d. (4 calves) with BHV2. The calves were given several i.v. inoculations of dexamethasone (DMS) and were killed at predetermined intervals subsequently. Attempts were made to isolate virus in tissue culture from nasal swabbings, nervous system tissues, lymph nodes and skin. Tissue were also examined for evidence of histopathological changes.

The immunologic relationship between BHV2 and HSV was studied in 15 Frisian calves, approximately 3 months of age and devoid of neutralizing antibody to either BHV2 or HSV. Five calves were injected with HSV1, fi-

ve with HSV2 and the remaining five calves were kept separated from the others as uninoculated controls. Either HSV1 and HSV2 inactivated antigens were inoculated to the calves in three times at intervals of 7 days. The first and the third inoculations were made intramuscularly with inoculums of 5 and 2.5 ml, respectively. The second inoculation consisted of 2.5 ml of antigen mixed with an equal volume of Freund's incomplete adjuvant, given by the subcutaneous route. Thirty-two days after HSV was first inoculated, all calves were subjected to challenge exposure with BHV2. The virus, in dilutions of 10^{-1} - 10^{-7}, was inoculated i.d. in the skin of the left side of the thorax of two calves in each group and of two control calves. Each diluted preparation in a volume of 0.2 ml was inoculated in quintuplicate. The remaining three calves in each group, and the other three control calves, were inoculated i.v. with 5 ml of undiluted virus. The calves were observed for a period of 25 days. Nasal swabs for virus recovery and blood samples for serologic tests were obtained from each calf one day prior to challenge, and on 3, 8 and 21 post challenge day (PCD).

RESULTS

Pathogenesis of the BHV2 infection

The results of the behavior of BHV2 in the target organs of the calf are shown in Table 1. Virus was first isolated, in the i.v. inoculated

TABLE 1 Recovery of BHV2 from experimentally infected calves.

Tissues	Virus isolation on post inoculation day from/to, from calves inoculated:		
	Intravenously	Intradermally	Intranasally
Skin	2/20	2/10	4/4
Nasal mucosa	NT	NT	4/6
Lymph nodes	3/7	Negative	Negative
Nervous system	3-5/7	2/6	2/6
Spinal fluid	5/7	Negative	Negative
Adrenal gland	2/2	NT	NT
Blood	3/3	Negative	Negative

NT = not tested.

calves, on post inoculation day (PID) 2 from skin and adrenal gland, 24 hours later it was detected in lymph nodes, blood and nervous system, and from the spinal fluid on PID 5. None of the calves killed through PID 5 was clinically affected. However, all those killed after PID 5 were febrile,had generalized skin lesions, enlargement of lymph nodes and were leukopenic. The i.n. exposed calves did not show any clinical signs of the disease. The virus was isolated from the nasal mucosa, the skin of the cheeks, and the trigeminal and glossopharyngeal nerves. In the case of the i.d. inoculated calves, virus was isolated from the skin of the inoculation site (left cheek) from PID 2-10 and from the trigeminal nerve, and the skin samples from the right cheek, perineum and scrotum, for a maximum of 6 days following infection. The scapular and precrural lymph nodes of these calves became enlarged, and a pruritus developed which was particularly intense in the skin of the scrotum and the thighs. The body temperature remained essentially within the normal range. Leukopenia was detected in some animals at PID 2-3; in others at PID 8-10. The histological changes induced by the virus in the skin consisted initially in the hydropic degeneration of the epithelial cells of the stratum spinosum, then all layers of the epidermis were involved with extensive epithelial necrosis: at this time the virus reached its maximum titer in the lesion. The main changes in the nervous system were the presence of intranuclear inclusions in the neural and glial cells

The results of the study on latency of BHV2 are reported in Table 2. The calves responded typically to BHV2 primary infection and all recovered in approximately three weeks time. Six months later, the calves were subjected to treatment with DMS. After corticosteroid treatment, signs of disease including leukopenia and enlargement of lymph nodes, which are characteristic of BHV2 infection, were observed. Virus was isolated from nasal swabbings of all animals 11 days following the DMS treatment. Virus was also isolated from skin, nervous system and lymph nodes of 1 calf. Following DMS treatment, a piroplasmal infection was demonstrated in this calf. Histopathological changes observed in the tissues were similar to

those already described for the primary infection with the virus.

TABLE 2 Reactivation in calves of BHV2 infection.

Calves No.	Infection	Fever	Leukopenia	Skin lesions	Virus isolation: Up to PID 10	From PID 12-180	After DMS treatment
Primary infection							
3	i.v.	Present	Present	D	+	-	NA
4	i.d.	Not present	Present	L	+	-	NA
After DMS treatment							
3	NA	Not present	Present	D	NA	NA	+
4	NA	Not present	Present	L	NA	NA	+

PID = post infection day. DMS = dexamethasone. NA = not applicable. D = disseminated or L = localized lesions. i.v. = intravenously. i.d. = intradermally.

Immunologic relationship between BHV2 and HSV

The response of calves to BHV2 challenge infection is depicted in Table 3. The evolution of the infection in the control calves was typical

TABLE 3 Clinical response of calves immunized with HSV to challenge exposure with BHV2.

Calves	BHV2 challenge exposure: No. of calves/ infection route	Days of: fever*	leukopenia*	Skin lesions: onset: PCD*	spreading
HSV-1 immune	2/i.d.	1	1	2	NA
HSV-2 immune	2/i.d.	1	0	2	NA
Controls	2/i.d.	2	3.5	2	NA
HSV-1 immune	3/i.v.	4.3	2.7	5.7	+
HSV-2 immune	3/i.v.	5	2.7	6.3	+
Controls	3/i.v.	7	6	6.3	+++

* Average. PCD = post challenge day. NA = not applicable. + = few lesions on limited area of the body (muzzle and chin). +++ = widespread, covering most of the body. i.d. = intradermally. i.v. = intravenously.

of BHV2 (Castrucci et al., 1972). The HSV-immune calves responded to BHV2

infection with clinical signs which were much less severe that those observed in the challenge control calves. The skin lesions in the calves exposed to i.v. inoculation of BHV2, were fewer in the calves in the HSV-immune groups, whereas they were heavily disseminated, covering the skin of the entire body, in the control calves. In the case of calves which received BHV2 i.d., the titer of the virus, as is shown in Table 4, underwent a reduction of more than one log unit in the pre-immunized calves.

TABLE 4 Intradermal titration of BHV2 in calves previously inoculated with HSV antigens.

Calves (No.)		ID_{50}* of BHV2 calculated on post-challenge day:		
		3	7	11
HSV-1 immune	(2)	4.28	5.60	5.40
HSV-2 immune	(2)	4.00	5.55	5.62
Controls	(2)	5.37	6.60	6.71

* Infective doses, neg. log.

BHV2 was recovered on PCD 3 and 8 from nasal swabbings of all HSV immunized or control calves. Samples obtained prior to challenge exposure or on PCD 21, were negative for virus.

The serum samples from the calves were tested for neutralizing antibody to either BHV2 or HSV according to the method reported elsewhere (Castrucci et al., 1984).

Thirty one days after the initial injection of HSV antigen, the calves showed an average antibody titer of 1:4 to the homologous immunizing HSV (HSV-1 immune calves), or 1:6 (HSV-2 immune calves), whereas there was no serologic response to BHV2 at that time by any of the calves (Table 5). Following challenge exposure with BHV2, the HSV antibody titer of the HSV-immune calves underwent a progressive increase, reaching a value of 1:8 on PCD 8 and 1:16 on PCD 21. Antibody to BHV2 was first detected in these calves on PCD 8 with a titer of 1:5; on PCD 21 titers of 1:32 and 1:60 were found in the calves immunized with HSV1 or HSV2, respectively.

In the control calves seroconversion to HSV was first detected on PCD 8. Seroconversion to BHV2 was evidenced in these calves only after 21 days of infection, at which time the titer was 1:20.

TABLE 5 Serologic response to HSV and BHV2 of calves immunized with HSV and subjected to challenge with BHV2.

Calves (No.)	Neutralizing antibody titers*							
	31 days after 1st HSV inoculation		After challenge with BHV2, on day					
			3		8		21	
	HSV	BHV2	HSV	BHV2	HSV	BHV2	HSV	BHV2
HSV-1 immune (5)	4	< 4	4	< 4	8	5	16	32
HSV-2 immune (5)	6	< 4	6	< 4	8	5	16	60
Controls (5)	NA	NA	< 4/< 4 °	< 4	4/4°	< 4	6/6°	20

* Average of the reciprocal value. ° Titer to HSV1/titer to HSV2.
NA = not applicable.

DISCUSSION

Studies of the behavior of BHV2 in the target organs of experimentally infected calves suggest that the pathogenesis of the infection most likely depends on the route of exposure to the virus. Thus, the i.v. inoculation (Castrucci et al., 1978) of the virus into calves, it produces a systemic infection, with generalized skin lesions and persistence of the virus in the skin. By contrast, a localized infection develops when the calves are exposed to the virus i.d. or i.n. (Castrucci et al., 1979). The study of the latency of BHV2 has confirmed that the virus, like other herpesviruses, can induce a latent infection in calves (Castrucci et al., 1982). On the basis of the virological and the histopathological findings, it seems reasonable to suggest that the skin, and probably the nervous system, are the host sites where the BHV2 persists in the latent form.

The immunologic relationship between BHV2 and HSV have been confirmed (Castrucci et al., 1984). Evidence of this is based on the clinical response, and the virologic and serologic findings made in the experimental calves. As compared to the control calves, which underwent a clinical re-

sponse typical of experimentally induced BHV2 infection, the HSV immunized calves showed a milder reaction to the challenge virus. The most significant virologic finding was made in the calves exposed i.d. to BHV2 infection. In these calves BHV2 titer underwent a drop of more than 1 log unit in the HSV-immune calves. In view of the serologic findings, it is interesting that HSV, while appearing to be unable to evoke antibody formation to BHV2, can reduce the latency period of the immuno-competent system of the host in producing antibody to BHV2. This hypothesis seems to be supported by the finding that the antibody titer to BHV2 at the end of the experiment (PCD 21), is higher in the serums of the HSV-immune calves than in the challenge infection control calves.

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Monoclonal antibodies against Bovine Herpesmammillitis Virus (BHV-2) and their cross reactivity with HSV-1 and -2

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Abstract

Monoclonal antibodies were prepared against BHV-2 infected cells. Some of their biological properties like neutralizing capacity and ability to recognize internal and external antigen structures of infected cells, were characterized. Based on the known cross reactivity of BHV-2, HSV-1 and -2, the human herpesviruses were included in these studies. At least 3 different classes of epitopes were defined: antigenic determinants common to BHV-2, HSV-1 and -2, antigens shared by BHV-1 and one of the human herpesviruses and those antigens only present on BHV-2.

INTRODUCTION

The serological relationship of the herpes simplex virus types 1 and 2 (HSV-1, HSV-2) and the bovine herpesvirus type 2 (BHV-2; Sterz et al., 1973; Ludwig, et al., 1978, Norrild et al., 1978; Yeo et al., 1980) offers a useful tool for the analysis of viral proteins. This is of particular interest since the common antigens induce cross-neutralizing antibodies. Analysis of the cross-reacting antigens revealed that common antigenic determinants are located on the viral glycoprotein gB of HSV (Norrild et al., 1978; Ludwig, 1983). It was shown that antisera directed against BHV-2 neutralize HSV much stronger than antisera directed against HSV do react with BHV-2 (Ludwig, 1983). The production of monoclonal antibodies directed against BHV-2 specific glycoproteins offers a possibility to study the expression of antigenic determinants on the cell surface of BHV-2 and HSV infected cells as well as on the viruses.

MATERIALS AND METHODS

Production of monoclonal antibodies

A31 cells, a permanent cell line derived from Balb/c mice were infected either with BHV-2

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(strain TVA) or HSV-1 (strain F) and used for immunization of Balb/c mice. Clones designated "C" are derived from a mouse immunized with BHV-2 infected cells, clones named "E" originated from a mouse immunized with BHV-2 infected cells and boosted with HSV-1 infected A31 cells. Clones were produced by fusion of NSI-Ag 4/1 myeloma cells with spleen cells of immunized mice following published procedures (Hybridoma techniques). After fusion the cells were suspended in medium and distributed in twenty 96-well microtitre plates.

Elisa

Screening of specific antibodies in hybridoma supernatants was performed on formaldehyde or glutaraldehyde fixed Georgia bovine kidney (GBK) cells infected with BHV-2 and on HSV infected African green monkey (Vero) cells grown in 96-well microtitre plates (NUNC, Denmark). Glutaraldehyde fixed infected cells were used to detect surface antigens, formaledhyde fixed Triton x-100 treated cells to monitor for internal antigens (Pauli et al., 1984).

Immunofluorescent assay

Vero cells were infected with BHV-2 (strain BMV). When the cpe was +++, the cells were removed from the petri dishes with 2 mM EDTA in PBS, washed once with PBS 5% fetal calf serum (FCS) and twice in PBS 1% FCS (washing buffer), followed by incubation with the monoclonal antibody for 1 hour at room temperature and two additional washings. The cells were then incubated with fluorescein isothiocyanate labelled anti-mouse IgG for 1 hour at room temperature, washed twice and fixed with 95% ethanol for 10 minutes, spun down and resuspended in 50% glycerol/PBS.

For the preparation of nuclei we essentially followed the procedure given by Tsutsui et al. (1983). BHV-2 infected cells were harvested, washed , suspended in 10 ml of RSB buffer (Tsutsui et al., 1983) and left for 5 minutes on ice. They were homogenized by 10 strokes in a glass homogenizer. The nuclei were centrifuged through a sucrose cushion (0.5 M sucrose in RSB) at 70 x g for 5 minutes and the pellet was resuspended in RSB buffer. Nonidet P-40 was added to a final concentration of 0.5% and the mixture homogenized by 10 strokes with a glass homogenizer. The homogenate was layered on top of a cushion (0.5 M sucrose in RSB) and centrifuged for 5 minutes at 70 x g. The pellet was resuspended in PBS and this nuclei-preparation was used for the immunofluorescent assays.

Neutralization test

Monoclonal antibodies were serially diluted in medium supplemented with 5% fetal calf serum, 0.5 ml of each antibody dilution were allowed to react with approx. 60-80 plaque forming units (pfu) of virus (BHV-2, strains BMV and TVA, HSV-1, strain F; HSV-2, strain G) for 10 min at 37°C. $2x10^5$ Vero cells in 100 µl of medium were added per well. 4 hours later the cells were overlaid with 0.5 ml medium containing 1.6% carboxymethylcellulose. After 36-48 hours the cells were fixed with 3% formaldehyde and stained with GIEMSA-solution.

To test the influence of complement the virus antibody mixtures were kept for 30 minutes at 37°C. Then rabbit complement was added (final concentration 1:30) and allowed to react for further 30 min at 37°C. Control experiments were performed with heat inactivated complement.

RESULTS

Monoclonal antibodies detecting antigenic sites in and on BHV-2 infected cells were selected for further studies. Such antibodies recognize antigens in the cytoplasm, on the nuclear membrane as well as in the cytoplasm (tables 1 and 2). Some of the antibodies crossreact with epitopes on HSV-1 or HSV-2 infected cells. The reaction patterns obtained in or on HSV infected cells were comparable to those seen in BHV-2 infected cells (table 1). Two different antibodies (C2D8, E23F12) were tested on BHV-2 infected unfixed cells or isolated nuclei. Both antibodies bind to antigens on the cell surface, whereas only the antibody staining the nuclei in fixed cells, reacted with antigens on the nuclear membrane (table 2).

clone	BHV-2 (GBK) s	BHV-2 (GBK) i	HSV-1 (Vero) s	HSV-1 (Vero) i	HSV-2 (Vero) s	HSV-2 (Vero) i
C2D8	+	c	+	c	+	c
E1D3	+	c	+	c	-	-
E18B11	+	c,n	+	c	-	-
E18E3	+	c	-	-	-	+
E22F12	+	c,n	+	c,n	+	c,n
E23F12	+	c,n	+	c,n	+	c,n
817-3	-	-	-	-	-	-

TABLE 1: Recognition pattern of monoclonal antibodies on or in fixed herpesvirus infected cells.
Recognition of antigen on glutaraldehyde fixed infected cells (s: surface, +: positive reaction; -: no reaction detectable) and distribution of antigens in infected cells (i: intracellular) after treatment of formaldehyde fixed cells with detergent (c: cytoplasm stained; n: nucleus stained; -: no reaction detectable).

clone	surface	nuclei
C2D8	+	+
E23F12	+	+
Q8H12	-	-

TABLE 2: Detection of antigens on the surface of unfixed BHV infected cells or nuclei isolated from infected cells
Uninfected cells or nuclei isolated from uninfected cells were not stained by the monoclonal antibodies. A monoclonal antibody (Q8H12) directed against surface antigens of Pseudorabies virus infected cells served as a control for the specificity of the reaction.

Neutralization tests performed with those monoclonal antibodies allowed a grouping in at least three categories (table 3): those neutralizing BHV-2, HSV-1 and -2 (C2D8), HSV-1 and BHV-2 (E18E3) and only BHV-2 (E1D3). One monoclonal antibody E23F12 was shown to enhance the infectivity of BHV-2 and HSV (up to two fold). In the presence of complement this antibody was able to inactivate BHV-2. This result implies that E23F12 antibodies react with epitopes on the surface of the viruses.

clone	IgG class	BHV-2	HSV-1	HSV-2	BHV-2+C'
C2D8	IgG1	++	++	++	-
E1D3	IgG1	+	-	-	-
E18B11	IgG2a	-	-	-	-
E18E3	IgG1	+	+	-	-
E22F12	IgG2b	-	-	-	n.t.
E23F12	IgG2b	e	e	e	+

TABLE 3: Reactivity of monoclonal antibodies with BHV-2, HSV-1 and HSV-2 in neutralization test.

++, + : neutralization of virus; -: no effect;e: virus titres are increased compared to controls or, monoclonal antibodies without neutralizing activity. Neutralization of BHV-2 was performed in the presence of rabbit complement (C', last column); +: neutralization in the presence of complement; -: complement independent neutralization; n.t.: not tested.

DISCUSSION

Monoclonal antibodies directed against BHV-2 specific antigens were selected on glutaraldehyde fixed cells. Such antibodies define antigenic determinants on BHV-2 proteins: first, epitopes which are conserved on BHV-2, HSV-1 and -2; second which are shared by BHV-2 and one of the human herpesviruses and third which are specific for BHV-2. The differentiation of epitopes bases on neutralization tests. These data together with the results obtained in the ELISA tests implicate, that the determinants might be expressed differently on the viruses and in infected cells. The monoclonal antibody E1D3, e.g., recognizes antigenic determinants in and on HSV-1 infected cells, but does not neutralize HSV-1, whereas the antibody reacts with BHV-2 antigens in fixed cells and inactivates BHV-2. Comparison of the biological activity of monoclonal

antibodies in neutralization tests and in the recognition of epitopes in or on the cell surface revealed that antibodies detecting antigen in the cytoplasm and on the cell surface can neutralize at least BHV-2, whereas an antibody recognizing also epitopes located on the nuclear membrane inactivates BHV-2 only in the presence of complement. Without complement this antibody (E23F12) enhances infectivity titres of the viruses. This effect might be due either to a stabilization of viral proteins or to a conformational change of the protein after antibody binding.

Preliminary biochemical analysis of the antigens recognized by the above described antibodies showed that the epitopes are localized on a BHV-2 specific glycoprotein which is recognized also by monoclonal antibody directed against gB (HSV-1) (Perreira et al., 1981).

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SUMMARY OF SESSION THREE.

IMMUNE RESPONSE TO HERPESVIRUSES IN CATTLE.

by G. CASTRUCCI and E. THIRY.

In this session four talks were given, the first two on Bovine herpesvirus 1 (BHV 1) and the other two on Bovine herpesvirus 2 (BHV 2). Two supplementary talks, given at the end of the seminar, were included in this session because their subjects were closely related to the other papers. One was consecrated to BHV 1, the other to Bovine herpesvirus 6 (BHV 6).

The first paper on BHV 1 was presented by P.-P. PASTORET (Belgium) and his group. The interaction between the specific immune response of cattle and the virus was considered, with particular attention being paid to the latent infection status of the animal. The events that characterize either the primary infection or the reactivation of a latent infection were described. The behaviour of both the unifected and the latently infected animals to vaccination was also depicted.

The author suggested an analytic method consisting of a series of equations the solution of which might give an answer of a reasonable reliability to the topic questions concerning the events mentioned above.

The second paper was presented by O.C. STRAUB (West Germany). The author reviewed and discussed the common serological diagnostic methods of BHV 1 infections in Europe. It was pointed out that all classes of immunoglobulins are able to react in classical serum neutralization tests whereas other tests, such as the ELISA or the RIA, can only detect antibodies belonging to the IgG group. It was also stated that the common serological tests are unable to differentiate between the various diseases caused by BHV 1; whether or not monoclonal antibodies could improve the serological diagnosis remains to be determined.

The paper of V. BITSCH (Denmark) dealt with control and eradication of infectious bovine rhinotracheitis which is under progress in Denmark. Specific and sensitive serological tests were developed by the author to allow the detection of infected herds and animals. Classical ELISA tests were considered to be as sensitive as the P37/24 virus neutralization test. However, an IBR blocking, direct ELISA test was also elaborated and found to be convenient by the author.

H. LUDWIG (West Germany) presented the paper of M. KAO on the spreading of goat herpesvirus (BHV 6) infection in several countries. It appeared that this infection is widespread and the existence of latent virus carriers is postulated in European and Asian countries.

The next paper, presented by G. CASTRUCCI (Italy), described some aspects of the pathogenesis of the infection induced by BHV 2 and also considered the immunologic relationship between this virus and Herpes simplex virus (HSV). Apparently, the pathogenesis of BHV 2 infection depends on the route of exposure to the virus: a systemic condition is produced when the virus is inoculated intravenously, whereas a localized disease is observed when the virus is inoculated through the intradermal or intranasal routes. From the immunologic relationship study it was proved that HSV partially protected calves against experimentally induced BHV 2 infection. The most significant serologic finding in this study was that HSV-preimmunized calves produced antibody to BHV 2 challenge virus at an earlier time than control calves.

The last paper of the session was presented by F. CONRATHS (West Germany). Monoclonal antibodies used in serum neutralization tests revealed the possibility of defining on a BHV 2 glycoprotein (M.W. 130,000) at least 3 epitopes, one of which shows cross-reaction with HSV 1 and HSV 2 (gB); a second antigenic site is shared by HSV 1 and HSV 2, whereas the third determinant seems to be specific for BHV 2.

Each presentation was followed by a stimulating discussion which proved very useful in giving a comprehensive meaning to the topic of the meeting.

SESSION 4

HERPESVIRUS PATHOGENICITY AND THE IMMUNE RESPONSE

Chairman : G. WITTMANN

Co-chairman : R.M. GASKELL

IMMUNITY AND PATHOGENESIS OF MALIGNANT CATARRHAL FEVER

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ABSTRACT

The two distinct causes of malignant catarrhal fever (MCF), alcelaphine herpesvirus-1 (AHV-1) and the as yet unidentified "sheep-associated" agent, produce no disease in their respective natural hosts, wildebeest and sheep, but cause fatal lymphoproliferative disease following transmission to other species of ruminant. Only with AHV-1 is there any substantial information on the immune response of the natural host to infection. Epidemiological studies indicate that all wildebeest become infected by 9 months of age and virus can be isolated periodically from some animals particularly pregnant females and young calves. The presumptive agent of sheep probably follows a similar epidemiological pattern.

The host immune mechanisms in wildebeest and sheep exquisitely ensure minimal detriment to the host yet exceptionally effective maintenance of the virus in even small animal communities. In complete contrast other species of ruminant infected by these viruses almost invariably die from catastrophic immunological dysfunction and virus does not normally spread to other animals.

Current research into the pathogenesis of MCF suggests that the disease represents a highly specific lesion of a subset of T-lymphocytes. Data which is being generated from studies of the disease in cattle, deer, rabbits, rats and hamsters suggest that the fundamental lesion in all cases is the same. Evidence is presented to support the concept that in MCF the large granular lymphocyte (LGL) represents an important target cell for the virus although expression is normally incomplete. It is proposed that virus induced dysfunction of these LGL results in both a profound polyclonal expansion of T-lymphocytes and the extension of natural killer cell activity to normal tissues which results in the widespread lymphoid cell hyperplasia and tissue necrosis which characterise MCF.

INTRODUCTION

Malignant catarrhal fever (MCF) is a generalised disease of ruminants caused by infection with either of two recognised agents (Plowright, 1968). In areas of Africa and in Zoological Parks around the world the disease occurs following transmission of alcelaphine herpesvirus-1 (AHV 1) (Plowright, Ferris and Scott, 1960) to cattle and certain other ruminant species from the natural host the wildebeest (Connochaetes spp.) (Reid and Buxton, 1984). The other agent, which has not yet been identified,

appears on circumstantial evidence to be a virus which normally infects sheep inapparently but which causes MCF on transmission to the other ruminants in particular cattle and deer (Reid, Buxton, Berrie, Pow and Finlayson, 1984). While neither agent produces any detectable clinical response in their respective natural hosts, in other species they both evoke the same dramatic reaction characterised by widespread necrosis particularly of the digestive and upper respiratory tracts and interstitial lymphoid cell accumulations in many tissues.

Current understanding of the pathogenesis of AHV1 infection in its natural host which is largely based on epidemiological studies of free-living populations and has recently been comprehensively reviewed (Plowright, 1984) will only be briefly summarised in this paper. The proposition that the "sheep-associated" agent behaves in sheep similarly to AHV1 in wildebeest is supported both by circumstantial evidence and by data suggesting that a virus sharing antigenic determinants with AHV1 is prevalent in sheep (Rossiter, 1981). Typical MCF caused by one or other agent has been reported in 11 species of Bovidae and 10 species of Cervidae as well as in laboratory rabbits, hamsters, rats and guinea pigs. Qualitatively the disease is the same in all species and is characterised by necrosis of the epithelia of the digestive and respiratory tracts as well as hyperplasia of lymph nodes and interstitial accumulations of lymphoid cells in many tissue. Although there are quantitative difference a common pathogenetic mechanism is assumed in this analysis.

A. RESPONSE OF THE NATURAL HOSTS

The recovery of virus from both fetal wildebeest spleen and from wildebeest calves under one week of age suggests that a proportion of wildebeest become infected in utero with AHV-1 (Plowright, 1965). Cell-free virus shed in nasal secretions (Mushi, Rossiter, Karstad and Jessett, 1980) would appear to be responsible for subsequent lateral spread - all wildebeest calves sero-convert by one year of age (Plowright, 1967). There is evidence that following infection, virus persists in the host and may be isolated intermittently from peripheral blood lymphocytes. Viraemia has been detected in 3/8 female wildebeest examined in the last months of pregnancy suggesting that recrudescence may occur at this time and allow spread of infection to the fetus. Although virus has generally been recovered from wildebeest leucocytes it should not be regarded as an obligate parasite of this cell type as it has been isolated from ocular and

nasal secretions, turbinate and corneal explant cultures and trypsin-dispersed kidney cell cultures (Mushi et al., 1980; Mushi, Rurangirwa and Karstad, 1981; Reid, unpublished).

No investigator has detected any clinical lesion attributable to infection and it is concluded that infection of wildebeest by AHV1 is normally benign. Antigenically related viruses would appear to behave in a similar fashion in two other species of antelope namely Coke's hartebeest (Alcelaphus buselaphus cokii) and topi (Damaliscus korrigum) although naturally occurring disease in other ruminants has never been attributed to these agents (Reid, Plowright and Rowe, 1975).

Rossiter (1981a) examined 167 sheep sera for antibody to AHV-1 using an indirect immunofluorescent test and neutralising antibody test. He was able to confirm that neutralising antibody was not present in sheep serum whereas 162 of the sera were positive for immunofluorescent antibody and the titres recorded were of the same order as found in sera collected from wildebeest. From this he concluded that a virus is prevalent in sheep which is similar to AHV-1 but which has at least one antigenic difference. Recent observations in this laboratory using AHV-1 antigen in a Western blot test to detect antibody in serum from several species support these findings. We have found that wildebeest sera react with several major bands and that all sheep sera tested react with a proportion of the same bands. There is therefore increasing evidence that there is a herpesvirus prevalent in sheep, antigenically related to AHV-1 which is considered to be the likely cause of sheep-associated MCF. This form of MCF in cattle and deer generally occurs in animals that have had contact with periparturient sheep (Reid and Buxton, 1984; Selman, Wiseman, Wright and Murray, 1978), implying that excretion of infective virus might be associated with this period, and lending support to the idea that the pathogenesis of both viruses in their normal hosts is similar.

B. RESPONSE OF OTHER RUMINANT SPECIES

Generally disease follows a prolonged incubation period of up to 6 months during which no obvious clinical signs can be detected. Onset of clinical disease is characterised by the abrupt development of fever with a generalised lymphadenopathy and inflammation and necrosis of the digestive and upper respiratory tracts and sometimes the skin. Conjunctivitis with corneal opacity progressing centripetally is frequently present and profuse lachrymal and nasal exudation rapidly becomes catarrhal. Meningo-

encephalitis, diarrhoea and dysentery can also occur. The clinical signs in all species caused by either agent are essentially the same although their duration, which may be peracute, acute or chronic, dictates the extent and severity of the lesions.

Virology and transmission

It is an extraordinary feature of MCF that despite the dramatic pathological changes there is an absence of evidence of virus-induced cytological changes in affected tissues and even infectivity may be difficult or impossible to demonstrate. Virus can however be consistently recovered from the blood leuocyctes of cattle reacting to AHV-1 and viraemia may be present for as long as 15 days prior to the onset of fever (Plowright, 1968). AHV-1 antigen has been identified in fewer than 2 cells per 10^6 of lymphoid tissues taken from infected calves, although infectivity as identified by isolation in tissue culture appears to be present in approximately 100-fold more cells (Patel and Edington, 1981). Recovery of AHV-1 whether in tissue culture or by animal inoculation requires viable cells and infection never spreads from infected cattle to in-contact animals. However, experimental transmission of this form of the disease from affected cattle or other ruminants to susceptible animals can readily be achieved using cells derived from lymphoid organs or blood (Plowright, 1968).

From cattle reacting with the sheep-associated agent no aetiological agent has been identified and even transmission from affected cattle to other cattle, with large volumes of blood and other tissues, cannot regularly be achieved (Plowright, 1968). In recent studies at our Institute large volumes of blood and lymphoid tissue suspensions were obtained from 18 individual clinical affected cattle cases of MCF and injected into calves, but on only two occasions was transmission successful. In marked contrast transmission from affected deer to other deer can be achieved with relative facility although to date, cattle inoculated with the same material have failed to react. Transmission of MCF to rabbits can also be readily achieved by inoculating tissue from affected deer (Buxton and Reid, 1980) but until recently all attempts to passage infectivity from cattle to laboratory animals had failed. However, at this laboratory each of two rabbits inoculated with a suspension of 10^9 lymph node cells from an affected cow reacted after 12 days with typical clinical and pathological signs of MCF while a calf given

10-fold more cells failed to react. It therefore appears likely that there are both quantitative and qualitative differences in the form of the sheep-associated agent present in different species and individuals at the time when clinical signs develop.

The immune response

Cattle infected with AHV-1 do not normally develop neutralising antibody but a proportion do develop antibody which can be detected by indirect immunoflurescence and immunoperoxidase methods and complement fixation tests (Rossiter, 1981b; Rossiter and Jessett, 1980). There is also evidence to suggest that some cattle infected with the sheep-associated agent may develop antibodies to AHV-1 which can be detected by fluorescence (Rossiter, 1983). However such antibodies do not appear to influence the fatal course of the disease. Furthermore, cattle hyperimmunised with tissue culture-propagated virus developed high titres of neutralising antibody but were still fully susceptible when challenged with virulent cell-associated virus (Plowright, Herniman, Jessett, Kalunda and Rampton, 1975). In an extensive study of AHV-1 in cattle approximately 6% survived experimental infection and 15/16 of the survivors were resistant to subsequent challenge (Plowright, 1968) suggesting that immunity to infection may develop in a few animals.

C. RESPONSE OF RODENTS AND LAGOMORPHS

Transmission of MCF caused by AHV-1 from affected cattle to rabbits was first reported in 1936 (Daubney and Hudson, 1936) and the resulting disease has subsequently been studied (Piercy, 1955; Edington, Patel, Russell and Plowright, 1979). Guinea-pigs, Syrian hamsters and rats also develop MCF following inoculation with AHV-1 and the disease can be transmitted through these laboratory species in series (unpublished data). Typical MCF can also be induced in rabbits and hamsters with the sheep-associated agent isolated from both affected deer and cattle. Following serial passage in rabbits AHV-1 and the sheep-associated agent may be transmitted to and passaged in rodent species but infectivity cannot apparently be passed back from rodents into rabbits. In addition AHV-1 which is readily recovered in tissue culture from affected rabbits cannot be recovered following passage in hamsters or rats suggesting that the virus may irreversibly adapt to these hosts. It is tempting to conclude that a similar adaptation might operate with the sheep-associated agent and explain some of the anomalous results of experimental attempts to transmit this agent from affected cattle and deer.

Virology

Several attempts to identify AHV-1 antigen in infected rabbits by immunofluoresence indicate that, as is seen in cattle, few cells express viral antigen. Such cells are limited to lymphoid tissues where they represent 1 to 4 cells per 10^6 (Patel and Edington, 1980; Edington and Patel, 1981). However, following short term culture of lymph node cells from affected rabbits the proportion of cells carrying viral antigen increased to something in the order of 1 cell per 10^3 (Patel and Edington, 1980). Whether this arises through expression of viral antigen in previously infected cells or by infection of cells occurring in vitro is not clear. Limited attempts to identify viral antigen in the rodent species infected with AHV1 have so far proved negative. Similarly, in rabbits infected with the sheep-associated agent no viral antigen has been identified by immunofluorescence using serum from cattle affected with this agent which gave positive fluorescence with AHV-1 antigen.

Immune response

The humoral immune response to viral antigen has been studied only in rabbits infected with AHV-1 (Rossiter, Mushi and Plowright, 1977). Neutralising antibody was detected in rabbit serum from about one day before the onset of pyrexia whereas fluorescent antibody could be detected as early as 4 days after infection. No rabbit has ever been observed to survive infection hence as in cattle the antibody reponse does not appear to contribute to recovery from the disease.

Both lymphocyte transformation tests and natural killer cell assays have been used to examine aspects of cellular immunity. The suggestion that the responsiveness of rabbit lymphocytes to B and T-cell mitogens is reduced during the course of infection (Wilks and Rossiter, 1978) has not be confirmed by subsequent workers (Russell, 1980). Natural killer cell activity in rabbits inoculated with the sheep-associated agent was investigated in this laboratory (Reid and Buxton, 1984). Rabbits were infected with a standard inoculum and individuals were examined at intervals. Target cells consisted of Cr^{51}-labelled rabbit kidney cell line (RK13) and effector cells were prepared from mesenteric and popliteal lymph nodes and spleens. No NK activity was detected in any control rabbit nor in clinically normal rabbits incubating the disease. However, from the first day of reaction and increasingly on the second and third days of reaction, high NK activity was present in all cell suspensions tested.

D. CULTURE OF LYMPHOCYTE CELL LINES

Until recently attempts to propagate lymphocytes from MCF-affected animals were unsuccessful. The first lymphoblastoid cell line to be described was designated MF120 and was obtained from a rabbit reacting with the sheep-associated agent (Reid, Buxton, Pow, Finlayson and Berrie, 1983). A suspension of mesenteric lymph node cells from the affected rabbit was mixed with fetal ovine kidney (FOK) cells and treated with polyethylene glycol before seeding in culture vessels. The resulting cultures had the appearance of normal monolayers of FOK cells and other cell types were not in evidence until day 21 in culture when the monolayer degenerated rapidly and numerous small round cells were observed in the supernatant fluid. These cells were maintained for several months in continuous culture by passaging them frequently to preformed monolayers of FOK cells as feeder cells. Recently they have been passaged on bovine turbinate feeder cells and it appears that they can be passaged indefinitely.

The MF120 cells have the rabbit karyotype and morphologically resemble large granular lymphocytes (LGL) an assessment confirmed by histochemical and ultrastructural examination. They were also cytotoxic to a variety of cultured primary cells and cell lines, a characteristic of natural killer cells, and it was concluded that they were a sub-population of lymphocytes known as natural killer LGL. Inoculation of rabbits with as few as 10^2 of these cells induced typical MCF indicating that they carry the agent although its identity remains obscure.

Subsequent to this, cells with the characteristics of LGL have been isolated from tissues of cattle (Berrie, Reid, Buxton, Pow and Finlayson, 1984) and deer (Reid, Buxton, Berrie, Pow and Finlayson, 1985) affected with the sheep-associated agent and from rats experimentally infected with AHV-1. They have been obtained from cultures of cerebrospinal fluid, cornea and lymph nodes. Typically a monolayer of fibro-epithelial cells forms which persists for days or weeks before suddenly degenerating, at which point numerous LGL can be detected in the supernatant fluid. Normally their continued culture is dependent on frequent passage to suitable feeder cell monolayers which are rapidly destroyed. The cytotoxicity displayed by these cells is characteristic of natural killer cells and it is apparent that like MF120 they are natural killer LGL. However unlike the MF120 cell line none of those derived from cattle or

deer has caused disease following inoculation into cattle, deer or rabbits. It is interesting that simian lymphocyte cell lines induced by Herpesvirus saimiri, a lymphotropic virus similar to AHV-1, required less than 27% of the viral genome to be present to maintain transformation (Schirm, Muller, Desrosiers and Fleckenstein, 1984). By analogy it is possible that the cell lines derived from cattle and deer affected with the sheep-associated agent may carry only a proportion of the viral genome which is insufficient to establish infection in recipient animals.

Cells characteristic of LGL with natural killer cell properties can also be obtained from both lymphoid and non-lymphoid organs of rats clinically reacting to AHV-1 (Reid, Jacoby, Pow and Buxton, 1984). These cell lines grow in clumps which appear in the supernatant fluid from the initiation of the culture and do not require feeder monolayers. Rats inoculated with these cells develop typical lymphoproliferative disease. However, it has not been possible to detect infective virus either by co-cultivation with permissive cell systems or by inoculation of rabbits. Thus, by analogy with the lymphoblastoid cell lines transformed by Herpesvirus saimiri, these rat cells may have conserved only a proportion of the AHV-1 genome.

Investigation of the possible role of interleukins in perpetuating the bovine and rat cell lines has been started. Pilot studies indicate that substances which significantly increase mitosis of normal bovine Concanavalin-A stimulated peripheral blood lymphocytes and an interleukin-2 (IL-2)-dependent rat cell line are present in the supernatant fluids of both types of cell. These results suggest that the production of endogenous IL-2 or similar lymphokine may have a role in the propogation of these cell lines.

E. PATHOGENESIS

1) Experimental studies

Critical studies of the pathogenesis of MCF have been confined largely to the experimental disease in rabbits (Edington et al., 1979; Buxton, Reid, Finlayson and Pow, 1984). Following inoculation of either agent there is a variable incubation period during which no overt clinical signs are present. An abrupt febrile response indicates the onset of clinical disease which consists of anorexia, depression and ocular and nasal discharges with death generally occurring on the second or third day. Terminal changes are characterised by widespread lymphoid hyperplasia with

follicular necrosis in lymphoid organs and extensive damage to the liver, respiratory and gastrointestinal tracts. In a study with AHV-1, follicular necrosis was observed as soon as 2-4 days following inoculation with a massive dose of lymph node cells from affected rabbits (Edington et al., 1979). These changes were not detected in rabbits killed between days 6 - 12 following inoculation although hyperplasia of the cortex of lymph nodes was observed. Severe lesions were seen in one of 2 rabbits killed on day 12 and in both killed on day 15. In a similar experiment using the sheep-associated agent (Buxton et al., 1984) the only early change observed was hyperplasia of lymphoid tissues which progressed throughout the incubation period while focal necrosis and degeneration were only observed in clinically affected animals (Figure 1). In these latter studies 5 x 10^7 lymph node cells from rabbits clinically affected with experimental MCF were used for inoculation. It is possible that the early necrotic lesions observed in the AHV-1 experiments could have arisen from a graft-versus-host or similar reaction resulting from the large inoculum employed and was not an integral feature of the MCF response. In the sheep-associated disease in rabbits the massive hyperplasia of lymphoid organs and lymphocyte accumulations in other tissues was shown to be due to an accumulation of T-lymphocytes. It is of interest that this T-lymphocyte hyperplasia was completely abrogated when cylosporin-A the potent T-lymphocyte suppressor (Borel, Feurer, Gubler and Stahelin, 1976) was administered daily to rabbits from the day before challenge and throughout the incubation period. These treated rabbits, however, succumbed with MCF after a similar incubation period to infected but untreated animals but the pathological changes which they developed were dramatically modified. The characteristic marked lymphoid hyperplasia and T-lymphocyte infiltration of non-lymphoid tissues was virtually ablated while necrosis in organs such as the liver was still present.

2) Hypothesis

A variety of pathogenetic pathways have been proposed to explain the dramatic changes seen in MCF. Plowright (1968) suggested that a hypersensitive response to viral or virus-induced antigens could be responsible. This concept was subsequently elaborated to include cell mediated responses to virus-infected vascular endothelium (Selman, Wiseman, Murray and Wright, 1974) or immune complex formation at membranes (Rweymamu, Mushi, Rowe and Karstad, 1976) or mechanisms analogous to graft

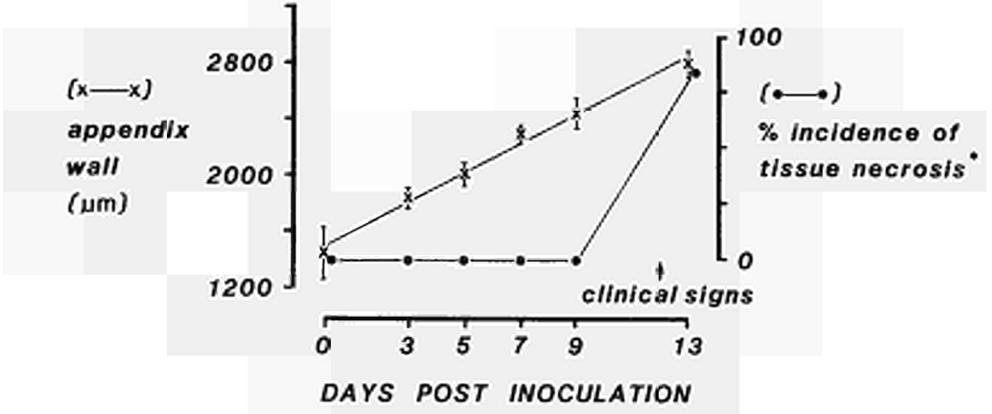

Figure 1. Development of pathology in rabbits experimentally infected with MCF. The mean (± SE) thickness (µm) of the wall of the appendix of animals killed at given times after the administration of a standard inoculum (Buxton et al, 1984) is shown (X——X). Each point represents the mean (± SE) of five measurements from each of two rabbits (three rabbits on day 13) made on formal fixed paraffin embedded sections stained by the Gordon and Sweet's method for reticulin.

The presence of tissue necrosis in the mesenteric lymph node, appendix, ileal Peyer's patch and the liver from each of the rabbits above is also shown (●——●). No attempt has been made to assess the amount of necrosis in a particular organ, thus the presence of necrosis in one of the four tissues examined from a particular rabbit would be expressed as 25 per cent.

Typical clinical signs of experimentally induced MCF in the rabbit were only seen on day 12 in the three rabbits killed on day 13.

rejection (Liggitt and De Martini, 1980). Virus-induced lymphoid cell transformation has also been postulated to occur (Hunt and Billups, 1979) but none of these explanations has proved entirely satisfactory. Reid and Buxton (1981) proposed that a profound virus-induced immune regulatory dysfunction could be the fundamental lesion and Denholm and Westbury (1982) suggested that a mechanism involving depletion of T-suppressor cells accompanied by a B-lymphocyte cytolysis could have a central role in the pathogenesis. However, the subsequent isolation and propagation of LGL with natural killer cell properties from a variety of tissues collected from rabbits, cattle and deer infected with the sheep-associated agent and rats infected with AHV1 suggest that these cells may have a central role in the pathogenesis of MCF.

We have suggested that LGL represent an important target cell for the viruses of MCF and that their subsequent dysfunction may have a central role in both the lymphoproliferative response and tissue destruction (Reid et al., 1985). We propose that IL-2 or similar lymphokine is produced in abundance by infected LGL and "drives" a T-lymphocyte hyperplasia, which is probably benign and polyclonal. The T-lymphocyte suppressor role that LGL are recognised to possess (Biron and Welsh, 1982; Trinchieri and Perussia, 1984) may also malfunction and contribute to the hyperplasia, possibly in concert with exuberant IL-2 production. We further suggest that tissue destruction which appears to represent a terminal event in the disease may arise from unrestricted natural killer activity of infected LGL causing cytolisis of normal cells.

The subtle variation in the distribution and severity of lesions in the different animal species affected with MCF and the widely varying incubation periods that are characteristic of the disease might thus be explained on the basis of the nature of the virus-induced lesions in NK cells. It should be noted that simian lymphoblastoid cell lines derived from Herpesvirus ateles and Herpesvirus saimiri also have characteristics of LGL (Johnson and Jondal, 1981) and the similarity between the pathology induced by these viruses and MCF has previously been stressed (Edington et al., 1979). The proposed pathogenesis for MCF may thus have wider biological implications.

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MAREK'S DISEASE HERPESVIRUS INFECTION
CHARACTERIZATION OF INFECTED TRANSFORMED T CELLS
BY THE USE OF MONOCLONAL ANTIBODIES

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SUMMARY

To obtain crude membrane extracts, thymic cells were harvested from thymus of 10 week-old SPF histocompatible chickens, and treated with 4 % Tween 40 supplemented Tris HCl Buffer Saline (T.B.S.). Nuclei were discarded. The supernatant was centrifuged and the pellet was purified by banding in discontinuous gradients of 10 to 48 % sucrose in 10 mM TBS. The highest major band was collected, pelleted and the protein content was measured by the Lowry technique.

These membrane extracts were used :
- for immunization in Balb C mice by 3 injections of 60 micrograms of T purified membrane protein extracts ;
- for antibodies detection by ELISA test in the supernatants of clone culture.

The fusion process was performed by contact between spleen cells of Balb C mice and SP2/0 Ag14 myeloma cells, at a ratio of 5:1 with polyethylene glycol. Fourteen monoclonal antibodies were specifically positive for thymocyte membrane extracts by Elisa test. Five out of the fourteen have been studied and were tested by indirect immunofluorescence against lymphoid cells from different origins. Three monoclonal antibodies react only with cells in both the cortical and medullary areas of the thymus. Two reacted with certain subpopulations of thymocytes, peripheral blood T lymphocytes, Marek's disease lymphoblastoid cell lines (MDCC-MSB_1, MDCC $HPRS_2$, MDCC RPL_1, MDCC-PA5, MDCC-PA9) and cells originating from MD tumors. These results demonstrated that MD cell lines and MD tumoral cells possessed a specific antigenic membrane determinant present only on mature T lymphoid cells.

INTRODUCTION

Marek's disease (MD) is a highly contagious disease of chickens caused by a herpes virus (MDV) (Churchill and Biggs, 1967). It is characterized by lymphoproliferation in the peripheral nerves and by the appearance of gross lymphomas in viscera, skin and other organs (Payne and Rennie, 1976). Tumors are composed of a variety of cells with a predominance of thymus-derived lymphoid cells (T cells).

Many lymphoblastoid cell lines have been established from MD lymphomas and all bear T cell markers (Powell et al., 1974 ; Nazerian and Sharma, 1975 ; Calnek et al., 1978). T cells have a dual role : they act as target cells for MDV transformation in susceptible chickens and participate in

immune surveillance against MD.

These functions could be carried out by different subclasses of T cells but our knowledge is much less documented in avian species than in mammals.

The production of monoclonal antibodies would be very useful for the identification and separation of lymphocyte subsets (Pink and Rijnbeek, 1983 ; Peault et al., 1982).

This work describes the production of monoclonal antibodies against different T cell surface antigens. Antibodies obtained were used to characterize different T cell subclasses and their distribution in some normal lymphoid tissues and in tumoral tissues from Marek's disease chicken tumors.

MATERIALS AND METHODS

Animals

Specific Pathogen Free B13/B13 homozygote chickens and BALB/c ByJ mice (Jackson laboratories, U.S.A.) were used for this work. Chickens were reared under S.P.F. conditions. One day old chickens were surgically bursectomized and used 10 weeks later as donors of thymic cells.

Preparation of thymic membranes

All procedures were performed at 4°C. Thymic lobes were disrupted with a tissue homogenizer. Cells were suspended at 5.10^5/ml in Tris/saline (T/s). They were crushed by magnetic stirring in 25 mM T/s added to an equal volume of 4 % Tween 40 in T/s. After centrifugation at 3,000 g for 30 mn, the pellet was discarded and the supernatant centrifuged for 60 mn at 70,000 g in a SW27 rotor on a Beckman L5-65 ultracentrifuge.

Membrane pellets were resuspended in 3 ml of 10 mM T/s layered on stepwise sucrose gradients. The steps used were 40,28 and 10 % sucrose (W/V). The gradients were centrifuged at 120,000 g for 16 hours. The lightest major band was collected, washed in 20 mM T/s to eliminate sucrose and concentrated by centrifugation at 120,000 g for 1 hour (Standring and Williams, 1978).

The protein content of the pellets was estimated by the Lowry technique (Lowry et al., 1951).

Purified extracts were kept at - 70°C.

Immunization of mice

Mice were injected 3 times with antigen (60 µg of purified membrane extracts) : the first of 30 µg IP and 30 µg IV, the second of 60 µg IV

15 days later and the third of 60 µg IV 20 days later. Mice were sacrificed 48 hours after the last injection and their spleen removed aseptically.

Fusion

Fusion was performed according to the general procedure (Köhler and Milstein, 1975) adapted by Grosclaude (personal communication).

Minimum Eagle Medium (MEM) supplemented with essential amino-acids and vitamins at double concentration was used for fusion. For culture, L glutamine (1 %), sodium pyruvate (1 %), penicillin (100 UI/ml), streptomycin (100 µg/ml) and 10 % foetal calf serum were added.

Spleen lymphocytes from mice were fused with myeloma cells $SP_2/0$ Ag14, at a ratio of 5:1. After 24 hours, SP2-like living cells, were dispensed at $8\text{-}12.10^4$ cells/0,4 ml in each well of culture plates.

One ml of selective medium (MEM with 10^{-4}M hypoxanthine, 10^{-5}M azaserin) containing 1.10^6 thymocytes from newly weaned mice added in the wells.

After 14 to 15 days, supernatants of growing hydrids which were at least 20 % confluent were removed and tested for specific antibody production.

ELISA test (Engwall and Perlmann, 1972)

ELISA test was used to detect hybrid cells secreting.

Reagents :

Alkaline phosphatase was used as enzyme-label for the antibodies and P-nitrophenyl disodium phosphate (PNPP, SIGMA) as substrate.

According to preliminary assays, 2 micrograms of protein crude membrane extracts were used as antigen for each well plastic plates (NUNC).

The wells were dried in hot air and incubated with hybridoma supernatants for 1 hour at 37°C. The supernatants were removed and the wells were washed 3 times with PBS containing 0,5 %. Tween 20. Rabbit anti-mouse IgG alkaline phosphatase conjugate (SIGMA) was added in each well at 1:1000 and the plates were incubated for 1 hour at 37°C. After 3 washings the substrate (10 % PNPP in diethanolamine buffer) were added in each well and the plates were incubated for 1 hour at 37°C. The enzymatic reaction was stopped with 4N NaOH. The coloration was measured in a semi-automatic Titertek Multiskan ELISA plate analyser.

When lymphoid cell suspension were used as antigen, the wells must be pretreated with L polylysine. Cells were fixed with glutaraldehyde at

0,025 % in PBS for 10 mn.

After 5 or 6 washings, gelatin was added. The plates were sealed and stored at 4°C for weeks or months until use (Lansdorp et al., 1980).

Marek's disease cell lines

The chicken lymphoblastoid cell lines used were MDCC-MSB1, MDCC $HPRS_2$, MDCC RPL1, MDCC PA5, MDCC PA9. The tissue culture medium was RPM1, 1640 with 10 % inactived foetal calf serum and antibiotics.

Indirect immunofluorescence test

Suspensions of 4.10^6 cells were incubated with 0,4 ml of monoclonal positive undiluted supernatants for 40 mn, rinsed twice in PBS and put in contact with rabbit anti-mouse Ig (NORDIC) at 1:20 for 40 mn at 4°C. After two further rinse, sheep anti-rabbit Ig labelled with fluorescein isothiocyanate (I. Pasteur, Paris) at 1:40 was added for 40 mn at 4°C. Cells were rinsed again, mounted on slides in buffered glycerin and observed with a Leitz Ortholux fluorescence microscope (Hudson and Roitt, 1973).

Immunoperoxydase test

Cell suspensions were fixed in 1 % paraformaldehyde in 0,85 M NaCl and washed in PBS. Samples of fixed cells were dried on slides at 4°C. Cells were incubated with monoclonal antibodies for 40 mn at room temperature, washed, incubated for 30 mn with Rabbit anti-mouse Ig conjugated with horse radish peroxydase and rinsed. Freshly prepared substrate (3'3' diaminobenzidine SIGMA) in phosphate buffer at pH 5 containing 0,02 ml/10 ml H^2O^2 was performed for 10 mn of contact. Slides were deshydrated and mounted in synthetic medium for microscopic observation (Kurstak et al., 1972).

RESULTS

Screening and selection of monoclonal antibodies

From initial 500 hybridomas proceeded, 60 were found to be positive by ELISA against purified T membrane extracts. Only 36 were selected for their stability and their competence upon passage and cloning. Some, also lost their capacity to secrete antibodies.

Among the 36 selected hybridomas, 14 were positive against chicken T membrane extracts, 12 were reacting with chicken T and B membrane extracts and 10 were cross reactive with chicken, duck and guinea fowl T membrane extracts (Table I).

Table 1 SPECIFICITIES ANTITHYMOCYTES MONOCLONAL ANTIBODIES

Examinated clones : 500 — Clones positive in Elisa test for antithymocytes : 60

Selected clones : 36

Specificity against	Chicken thymocytes	Chicken thymocytes and bursacytes	Chicken, duck and guinea-fowl thymocytes
Positive clones	14	12	10

Properties of some monoclonal antibodies

Five out of the fourteen hybridomas specifically reacting with chicken thymocytes were tested against different lymphoid cells.

Results are summarized in tables 2 and 3.

Three monoclonal antibodies (8B6, 9B1, 24A3) belonging to the IgG1 subclass, reacted only with cells in both the cortical and medullary areas of the thymus. They did not react with bursacyte membrane extracts and a low reactivity (< 10 %) was noted against whole cells from bursa. This may be due to the normal presence of a few T cells in the bursa (Toivanen et al., 1981). They neither reacted against peripheral blood lymphocytes nor cells of Marek's disease cell lines or MD tumor cells from chickens.

The two other monoclonal antibodies (8C4, 9A1) belonging to the IgG2a subclass, were positive with peripheral blood lymphocytes, and MD lymphoma cells. Whereas 8C4 reacted with all tested MD cell lines, 9A1 was only positive with certain MD cell lines.

Table 2 SPECIFICITIES OF MONOCLONAL ANTIBODIES AGAINST NORMAL LYMPHOID CELLS

Antigens \ MA	8B6	9B1	24A3	8C4	9A1
Thymocyte Membrane (b) Extracts	+++ (a)	+++	+++	+	++
Bursacyte Membrane (b) Extracts	-	-	-	-	-
(c) Thymocytes	+++	+++	+++	+	++
(c) Bursacytes	+ < 10 %	+ < 10 %	+ < 10 %	N.T.	N.T.
(b) Blood lymphocytes	-	-	-	++	+

(a)+,++,+++ Intensity of response (b) Elisa test (c) Im. Fluor. test
N.T. Not tested

Table 3 SPECIFICITIES OF MONOCLONAL ANTIBODIES AGAINST MD CELL LINES AND MD TUMORAL CELLS

Antigens \ MA	8B6	9B1	24A3	8C4	9A1
(a) MD cell lines				(c)	
MDCC RPL 1	-	-	-	100 %	100 %
" MSB 1	-	-	-	"	0 %
" HPRS 2	-	-	-	"	50 %
" PA 9	-	-	-	"	10 %
" PA5	-	-	-	"	10 %
(b) MD Tumoral cells					
Liver	-	-	-	100 %	100 %
Ovary	-	-	-	"	"
Kidney	-	-	-	"	"
Testis	-	-	-	"	"

(a) Immunofluorescence or peroxydase test (b) Immunofluorescence test
(c) % marked cells

DISCUSSION

This report studies the characterization of five monoclonal antibodies directed against thymic cells from chickens. Three of them 8B6, 9B1, 24A3 did not bind to lymphocytes of peripheral blood. On the contrary 8C4 and 9A1 recognized also certain lymphoid cells of blood.

These results suggest the existence of, at least, one antigenic determinant common to peripheral blood and thymic lymphocytes. This could correspond to an antigen appearing late in the maturation of T cells.

On the other hand 8B6, 9B1, 24A3 reacted with epitopes present only on the majority of thymic lymphoid cells. This response was not related to a particular chicken line sex or age, which could mean that these antibodies are specific for T cells of the thymus.

On tumoral cells authors have described the presence or the absence of different information on the MD cell line membranes (Ross et al., 1977 ; Coleman et al., 1980 ; Powell et al., 1974 ; Witter et al., 1975 ; Schat et al., 1982).

Our finding that 8B6, 9B1, 24A3 tested by immunofluorescence did not label cells derived from tumors and MD cell lines, show that these specific thymic epitopes cannot be detected on tumoral cells. However, 8C4 and 9A1 labelled both blood lymphoid cells and tumor derived cells. This result could suggest that the tumors are composed either of cells having reached a certain degree of maturity or of lymphoid cell lines

expressing early a specific membrane antigenic determinant characteristic of later stages of the differentiation process.

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IMMUNITY TO AUJESKY'S DISEASE VIRUS IN PIGS

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ABSTRACT

A survey on the present knowledge of humoral and cell-mediated immunity to ADV is given on account of experimental datas. The following parameters of immunity have been investigated: Neutralizing antibodies (Ab), spontaneous cell-mediated cytotoxicity against non-infected (SCC) and ADV-infected target cells (ADV-SCC), antibody-dependent cell-mediated cytotoxicity (ADV-ADCC), lymphocyte stimulation (ADV-LYST) and interferon production. No datas have been available with regard to T-cell cytotoxicity.

The state of immunity before and after ADV infection is described with unvaccinated and vaccinated pigs without and with colostral antibodies and with latently infected pigs. The evaluation of the results has been rather difficult since no uniform reactivity of the animals existed and great individual variations occurred.

Regardless of the pre-infectious immune state of the animals no enhanced immunological reactivity could be found during the first 4 days after infection. In the contrary, pigs with severe clinical symptoms showed depressed ADV-SCC. Enhanced ADV-SCC, ADV-LYST and Ab production occurred between DPI 7 and DPI 14. ADV-ADCC appeared in animals without pre-infectious Ab on DPI 14, when IgG-Ab were predominant. Discernible relations to the course of the disease did not exist with neutralizing Ab, ADV-ADCC, ADV-LYST and interferon production.

No significant influence on SCC and ADV-SCC could be detected after vaccination, whereas ADV-ADCC, ADV-LYST and Ab production were initiated and increased after the second vaccination. Ab production after primary vaccination was inhibited by colostral Ab, but a booster effect occurred after the second vaccination. This was also reflected in ADV-ADCC.

Latently infected pigs displayed SCC, ADV-SCC, ADV-ADCC and ADV-LYST. Neutralizing Ab could be demonstrated in all the animals tested between 3 and 22.5 months after ADV infection. After immunosuppression significant reduction of SCC and ADV-LYST occurred whereas neutralizing Ab titres generally were not reduced. In the contrary, an increase of ND_{50} values occurred after 2-3 weeks.

The results are discussed with regard to relations between the different immunological parameters and the course of ADV infection or protection, respectively.

ABBREVIATIONS

Ab: antibodies, ADV: Aujeszky's disease virus, ADV-ADCC: ADV specific antibody-dependent cell-mediated cytotoxicity, ADV-SCC: ADV specific spontaneous cell-mediated cytotoxicity, ADV-LYST: ADV specific lymphocyte stimulation, CMI: cell-mediated immunity, cpm: counts per minute, CTL: cytotoxic T lymphocytes, N-ADCC: non-ADV specific ADCC, SCC: spontaneous non-specific cell-mediated cytotoxicity, WBC: White blood cells.

INTRODUCTION

Humoral immunity and CMI are considered as important factors in the immune defense mechanisms against ADV. Whereas humoral immunity is well known for years, CMI is being investigated only for a few years. It was shown that ADV induces reactions of CMI: ADV specific lymphocyte stimulation (Van Oirschot, 1978/1979; Wittmann et al., 1976), inhibition and enhancement of macrophage migration (Wittmann, 1976), enhancement of leukocyte migration (Gutekunst, 1979), enhanced spontaneous cell-mediated cytotoxicity against ADV infected target cells (Martin and Wardley, 1984; Wittmann et al., 1983; Wittmann and Ohlinger, 1984) and reactivity of ADV-antibodies in ADCC (Ashworth et al., 1979; Wittmann et al., 1983; Wittmann and Ohlinger, 1984). In vivo, cutaneous hypersensitivity of the delayed type occurs (Skoda et al., 1968; Smith and Mengeling, 1977).

We are working on immunity against ADV for some years with priority to CMI. We investigated the immunological response of pigs after infection and after vaccination and infection, the response of colostral immune pigs to infection and to vaccination and infection, and the immunological state of latently ADV infected pigs. I want to present a survey of our results, a part of which has been published (Wittmann et al., 1983; Wittmann and Ohlinger, 1984) or is being published (Wittmann et al., 1985).

MATERIALS AND METHODS

Details of the techniques used have been published. The neutralization test was done in the presence of guinea pig complement which enhances neutralization, especially with IgM antibodies (Jakubik and Wittmann, 1978). For LYST we used the macromethod with unpurified WBC and unpurified inactivated ADV and control antigen at the beginning (Wittmann et al., 1976). Later on we used the micromethod with Ficoll-purified lymphocytes and partly purified inactivated antigens (Wittmann and Ohlinger, 1984). The cell-mediated cytotoxicity tests (Wittmann and Ohlinger, 1984) were usually performed with WBC as effector cells and ^{51}Cr labelled Vero cells as targets. The effector target cell ratio was 100:1 and the incubation time was 17 hrs at 37°C with all the tests. These conditions were found to be optimal.

The tests were done in triplicates. The cytotoxicity indices were determined (in per cent) according to the formula

$$\frac{\text{cpm test-cpm spontaneous } ^{51}Cr \text{ release}}{\text{cpm maximal } ^{51}Cr \text{ release-cpm spontaneous } ^{51}Cr \text{ release}} \times 100.$$

The following parameters of cell-mediated cytotoxicity were checked:

Spontaneous cell-mediated cytotoxicity against non-ADV infected target cells (SCC) and against ADV infected target cells (ADV-SCC). By treatment of the effector cells with monoclonal Ab against porcine CTL (Jonjic and Koszinowski, 1984) we could demonstrate that the SCC active lymphocyte population(s) were non-T lymphocytes which were non-adherent and very active against K 562 target cells. From this it can be concluded that they were NK-cells. The possibility that ADV-SCC reflected ADCC evoked by in vivo adsorbed Ab could be excluded since 37°C-treatment followed by repeated washings, procedures which cause elution of adsorbed Ab (Greenberg et al., 1977; Hoh and Babiuk, 1979), did not reduce ADC-SCC activity.

For antibody-dependent cell-mediated cytotoxicity against ADV infected target cells (ADV-ADCC) Ab and effector cells from the same animal were used at the time of testing. As control for non-ADV specific ADCC (N-ADCC), e.g. ADCC evoked by Ab against non-viral vaccine constituents, non-ADV infected target cells were employed.

It was found that the whole WBC fraction was essential for ADCC, since ADCC activity was significantly reduced when purified lymphocytes were used instead of WBC. However, no further reduction occurred, when the lymphocyte preparation was treated with monoclonal Ab against CTL. This indicates that the lymphocytes which participate in ADCC were non-T cells. These results correspond very well to the results of Ashworth et al. (1979), who found that neutrophilic granulocytes and monocytes/macrophages were the most active cell types in ADCC.

We could not examine T-cell cytotoxicity since we did not have a syngenic system which seems to be necessary for detection of CTL.

The statistical methods used for determination of the significance of positive results in the different tests have been described (Wittmann and Ohlinger, 1984). On account of multiple tests cytotoxicity values of $\geq$11% above cell controls in SCC were significant ($p \leq 0.01$). ADV-SCC, that is enhanced SCC against ADV-infected targets, is considered as significant on account of the least significant differences between two individual index values when the ADV-SCC cytotoxicity value was $\geq$13.3% above the corresponding SCC cytotoxicity value. With the same calculation ADV-ADCC is significant when its cytotoxicity value exceeds that of SCC by 17.7%, that of ADV-SCC by 18.7% and that of N-ADCC by 17.7%. In some cases the ADV-SCC intensity was so high that ADV-ADCC could not significantly exceed it. The significance of the comparison of the number of positive animals in 2 groups was calculated on the 90% level by the fourfold table test.

TABLE 1 Immunological behaviour of non-immune pigs after infection.

	DPI					
	0	2-4	6-9	14-18	42	84
SCC	11/26[a)] 42.3%	14/26 53.8%	13/22 59.1%	19/22 86.3%	7/8 87.5%	5/8 62.5%
ADV-SCC	6/26 23.1%	5/26 19.2%	9/22 40.9%	8/18 44.4%	3/8 37.5%	3/8 37.5%
ADV-ADCC	0/26 0%	1/26 3.8%	1/22 4.5%	21/22 95.5%	8/8 100%	8/8 100%
ADV-LYST	0/26 0%	4/26 15.4%	17/22 77.3%	22/22 100%	8/8 100%	5/8 62.5%
ND_{50}	<2	1.7[b)]	8/DPI 6[c)] 133/DPI 7-9[d)]	188	99	160

a) Positive/number examined
Underlined significant differences ($p \leq 0.1$) with regard to day 0

b) Eight of 26 pigs with positive ND_{50} (mean 1:5.6) on DPI 4

c) Mean ND_{50} on DPI 6: 1:8

d) Mean ND_{50} on DPI 7-9: 1:133

Significance of ADV-LYST in comparison to the controls and to LYST by control antigen was determined by the t-test.

The interferon assay of the sera was done in one experiment according to the method described earlier (Wittmann et al., 1980).

RESULTS

Before the results of the experiments are presented general remarks concerning the results are necessary. The animals did not show uniform reactivity since great individual variations occurred. For example, an animal had shown SCC, 3 weeks afterwards it was negative, and after further 2 weeks it was positive again. The same was true with ADV-SCC, ADV-LYST and to a considerably less degree with ADV-ADCC. This means, that when we get a ratio of positive animals on different days some animals which were positive on the one day were negative on the other day and vice versa. Therefore, it was impossible to interpret the results of CMI tests on the base of individual animals and rather difficult to do this with groups of animals.

For determination of the cytotoxic potential of normal pigs we examined 49 animals between 11 days and 7 months old, but the majority was between 3 and 12 weeks old. In total, SCC by WBC was found with 49.0% of the animals and ADV-SCC with 24.5%. No ADV-ADCC, ADV-LYST and neutralizing ADV-Ab could be detected.

Twenty-six of these animals were ADV infected when 12 weeks or 7 months old. The immunological behaviour after infection is shown in Table 1.

SCC was detected in 42.3% and ADV-SCC, in 23.1% of the animals on DPI 0. After infection an increase occurred from DPI 2 to DPI 4 onward, and 86.3% of the animals were positive in SCC and 44.4% in ADV-SCC on DPI 14.

ADV-ADCC was regularly detected from DPI 14 onward. This corresponds to the results of Martin et al. (1983). But neutralizing Ab were already present in considerable amounts on DPI 7. These early Ab were of the IgM class and complement

TABLE 2 Relation between clinical symptoms and immunity.

Group		DPI					
		0	4	6	7	8/9	14/18
I	SCC	2/6	3/6	2/2		2/2	1/1
Very severe	ADV-SCC	0/6	0/6*	0/2*		0/2	0/2
clinical	ADV-ADCC	0/6	0/6	0/2		2/2	2/2
symptoms,	ADV-LYST	0/6	1/6	2/2		2/2	2/2
died	ND_{50}	<2	<2	5		450	437
	IF^{50}	<4	17	<4			
II	SCC	5/16	7/16	7/8		14/16	12/16
Severe	ADV-SCC	3/16	4/16*	0/8*		15/16	10/16
clinical	ADV-ADCC	0/16	1/16	0/8		6/16	16/16
symptoms	ADV-LYST	0/16	3/16	8/8		11/16	16/16
	ND_{50}	<2	3	9		164	187
	IF^{50}	<4	20	<4			
III	SCC	4/4	4/4		4/4	4/4	2/4
Weak	ADV-SCC	1/4	1/4		1/4	3/4	2/4
clinical	ADV-ADCC	0/4	0/4		0/4	1/4	3/4
symptoms	ADV-LYST	0/4	0/4		4/4	4/4	4/4
	ND_{50}	<2	<2		238	238	151
	IF^{50}	<4	14		28(2)[a]	14(1)[a]	<4

* Significant reduction of cytotoxic activity of the WBC in comparison to SCC with a part of the negative animals

IF: Inferferon titre

a) Number of positive animals

requiring. But ADCC is a function of IgG, which are the predominant Ab-class on DPI 14 and later. Since in the bovine system IgM react in ADCC in the presence of complement (Rouse et al., 1977) we added in some instances guinea pig complement to ADCC-tests with early sera, however, no positive ADCC occurred. Pig complement was unsuitable because it was cytolytic for the target cells. But this does not exclude that IgM-ADCC in pigs might occur in vivo in the isogenic system.

ADV-LYST was first detected on DPI 4 with a few animals. The number of positive pigs rose to 77.3% on DPI 6/DPI 7, and to 100% on DPI 14.

Neutralizing Ab were demonstrated with 8 pigs in very low titres on DPI 4 and with most of the pigs on DPI 6. A significant rise of the titres appeared on DPI 7 and the maximal level was reached on DPI 14. Then the titres remained rather unaltered until DPI 84.

In this connection I want to say that in other long term experiments we could never find that neutralizing Ab disappeared during the course of 2.5 years. In the contrary, the titres remained rather high with most of the animals. This may be due to reactivation of the latent virus.

From the infection experiment the question arises whether relations existed between the clinical course of the disease and the immune response. This is difficult to answer and I have compiled the results in Table 2 with regard to this question.

In all the three clinical groups no significant differences could be found in Ab production, SCC, ADV-SCC, ADV-ADCC and ADV-LYST with regard to the number of reacting animals. With ADV-SCC, however, differences existed with regard to the intensity of cytotoxicity on DPI 4. The mean cytotoxicity index, which is not given in the table, of group I was 2.9%, of group II 10.3% and of group III 43.5%.

Further, it was observed that with 5 of the 6 animals of group I the ADV-SCC cytotoxicity index was significantly reduced in comparison with the SCC cytotoxicity index, and

TABLE 3 Immunological behaviour of pigs after vaccination and infection.

	DPV				DPI			
	First vacc.		Second vacc.					
	0	21	7	14	4	7	14	150
SCC	12/18 66.7%	12/18 66.7%	18/18 100%	7/18 38.9%	9/9 100%	14/18 72.8%	18/18 100%	16/17 94.1%
ADV-SCC	2/18 11.1%	5/18* 27.2%	7/18* 38.9%	9/18 50%	7/9 77.5%	15/18 83.3%	8/18 44.4%	4/17 23.5%
ADV-ADCC	0/18 0%	8/18 44.4%	17/18 94.4%	18/18 100%	9/9 100%	17/18 94.4%	18/18 100%	16/17 94.1%
ADV-LYST	0/18 0%	6/18 33.3%	8/18 44.4%	5/18 27.1%	9/9 100%	18/18 100%	14/18 77.8%	9/17 52.9%
ND_{50}	<2	43	224	190	540	1139	1216	198
Symptoms						Fever		

* Significant reduction of cytotoxic activity of the WBC in comparison to SCC with a part of the negative animals

Underlined significant differences ($p \leq 0,1$) with regard to day 0

the cpm in ADV-SCC lay frequently beneath the cmp of the cell control. In group II 5 of the 16 animals reacted in the same way. This means that in some instances the NK-cells had completely lost their cytotoxic activity against ADV infected target cells.

The reason for this depression is not known. One could argue that ADV multiplication had occurred in the cytotoxic effector cells and destroyed them, or that ADV infected Vero cells were unable to bind NK-cells as it has been described with herpes simplex virus (Welsh and Hallenbeck, 1980). However, these events would have occurred at every time and not only on DPI 4/DPI 6. A further possibility would be that target cells were rendered insusceptible against the cytotoxic activity of NK-cells by interferon (Trinchieri and Perussia, 1981/82). However, Vero cells do not produce interferon (Welsh and Hallenbeck, 1980), and if interferon producing effector cells were present, the depressor effect of interferon must also have been evoked in SCC.

Thus, it might be that NK cell activity against ADV infected cells plays a role in the early phase of ADV infection. For the interpretation of the results it must be considered that the number of animals in group I and III was very small. Thus, our consideration with regard to ADV-SCC are statistically not secured.

With regard to interferon type I (ß-IF) no differences existed between the pig groups on DPI 2 (not given in the Table) and DPI 4. However, 2 animals of group III showed prolonged IF titres on DPI 7 and one animal on DPI 9.

The immunological behaviour of pigs after vaccination and infection is summarized in Table 3. At the time of vaccination with an inactivated vaccine the pigs were 11 weeks old. Revaccination was done 21 days after the first vaccination and infection 14 days afterwards.

SCC was detected in 66.7% and ADV-SCC in 11.1% of the animals on day 0. After vaccination SCC remained rather unaltered whereas ADV-SCC increased up to DPRV 14. A part of the animals showed reduced ADV-SCC cytotoxic activity of the

WBC after the first vaccination and 7 days after the second vaccination. Both SCC and ADV-SCC increased after infection, but the increase in SCC was not significant, whereas the increase of ADV-SCC was significant on DPI 7 but a decrease occurred on DPI 14.

ADV-ADCC occurred with 44.4% of the pigs after the first vaccination and with 100% after the second vaccination. This level was maintained after infection.

ADV-LYST was detected in about one third of the animals after vaccination. It did not increase after revaccination, however, after infection all the animals were positive during the first week and 77.8% after the second week p.inf.

By means of ADCC with non-infected target cells and by LYST with control antigen we could demonstrate that immunological reactivity against non-viral vaccine constituents existed in the vaccinated animals.

Neutralizing Ab were present with a mean titre of 1:43 after the first vaccination and after the second vaccination the titre rose to about 1:200. Infection evoked a marked booster effect on DPI 4 (titre 1:540) and maximal values on DPI 7 and DPI 14 (titre about 1:1200). After 150 days the titre dropped to about 1:200.

After infection the vaccinated animals did not show clinical signs of AD, but 77.8% of the pigs reacted with fever. However, no significant relations were found between the occurrence of fever and the immunological response.

By comparing the immunological behaviour of non-vaccinated and vaccinated animals after infection it could be found: Both groups showed a tendency of increased SCC. ADV-SCC significantly increased only with the vaccinated animals during the first week p.inf., but decreased after the second week, reaching a level like with the non-vaccinated animals, ADV-ADCC was present in the vaccinated animals at the time of infection, but in the non-vaccinated pigs it lasted two weeks until ADV-ADCC was regularly detected. ADV-LYST was present in all the vaccinated pigs on DPI 4 and later on, but with the non-vaccinated pigs it tooks until DPI

TABLE 4 Immunological behaviour of latently infected pigs (3-22,5 months p.inf.).

Immuno-suppression	SCC	ADV-SCC	ADV-ADCC	ADV-LYST	ND_{50}	Virus recovery
No	8/12 66.7%	5/12 41.7%	12/12 100%	8/12 66.7%	184	4/12
Before	7/11 63.6%	2/11 18.2%	11/11 100%	8/11 72.7%	168	
3-4 days after IS	2/11 18.2%	1/11 9.1%	11/11 100%	1/11 9.1%	138	8/11

Underlined significant reduction

7 to reach this level. The production of neutralizing Ab was boostered in the vaccinated pigs on DPI 4, whereas low levels of Ab were detected in all the non-vaccinated pigs only on DPI 6 and the maximum level was reached on DPI 14. The Ab level in the vaccinated infected pigs were about 7-times higher than in the non-vaccinated pigs.

Thus, protection against ADV infection seems to be predominantly a matter of neutralizing Ab and eventually of ADCC. However, the participation of enhanced spontaneous cell-mediated cytotoxicity against ADV-infected cells (ADV-SCC) during the first week of infection may act as a further defense mechanism.

After the recovery phase the AD virions disappear and only virus DNA persists latently in certain parts of the body. The immunological state of latently infected pigs is shown in Table 4. The animals, most of them vaccinated, were tested between 3 and 22,5 months after ADV infection. The recovery of the virus was performed by co-cultivation of different tissues in MDBK cells.

SCC was displayed by 65.2% of the pigs, ADV-SCC by 30.4%, ADV-ADCC by 100% and ADV-LYST by 69.6%. Neutralization titres ranged from 1:14 to 1:512 without respect of time. Titres between 1:128 and 1:512 were even found after 22,5 months. This might be an indication that spontaneous reactivation of the latent virus occurred during latency.

The effect of immunosuppression on the immune response of the animals was mainly pronounced in SCC and ADV-LYST. The rate of SCC was significantly reduced from 63.3% of reacting animals before IS to 18.2% after this treatment, and that of ADV-LYST was significantly reduced from 72.7% to 9.1% of reacting animals. In contrast, IS did not alter ADV-ADCC. This may be in connection with the fact, that the neutralizing antibody titres in general remained unaltered after IS. A significant reduction only occurred in 2 of 13 pigs, which were tested on day one after the end of IS. However, in other experiments it could be demonstrated that a significant increase of ND_{50} values occurred with most of the

TABLE 5 Immunological behaviour of piglets with colostral antibodies before and after infection.

	DPP			WPP	DPI		
	1-2	5-6	9-12	7-9	2-4	7	14
SCC	2/8 25%	0/8 0%	1/7 14.3%	8/13 61.5%	4/11 36.7%	11/11 100%	5/11 45.5%
ADV-SCC	1/8 12.5%	0/8 0%	1/7 14.3%	6/13 46.2%	4/11 36.4%	4/11 36.4%	10/11 90.9%
ADV-ADCC	7/8 87.5%	4/8 50%	4/7 57.1%	2/13 15.4%	2/11 18.2%	1/11 9.1%	10/11 90.9%
ADV-LYST	0/6 0%	0/8 0%	0/7 0%	0/6 0%	0/4	4/4	5/11 45.5%
ND_{50}	77	112	106	62	67	166	181
Symptoms						Fever	

Underlined significant in comparison to WPP 7-9

animals 2 to 3 weeks after immunosuppression (Wittmann et al., 1983).

It remains to be clarified whether the reduction of SCC and of sensitized lymphocytes ADV-LYST are in connection with the reactivation of the latent virus. Neutralizing serum Ab were apparently not involved in this process. This is not surprising, when the virus is distributed on neural pathways and in lymphocytes and macrophages.

The next point examined was the immunological behaviour of pigs with colostral antibodies before and after infection (see Table 5).

SCC and ADV-SCC were present in some of the piglets from DPP 1 to DPP 12. The number of positive animals increased at WPP 7 to 9, reaching the usual level of older animals. This may be an indication that SCC activity develops only after birth, what coincidents with results of other investigators (Kim et al., 1980; Cepica and Derbyshire, 1984), who did not detect NK-cell activity in pig fetuses. After infection SCC increased on DPI 7 and ADV-SCC on DPI 14, however, these increases were not significant with regard to DPI 0.

ADV-ADCC was present in 87.5% of the animals on DPP 1 and DPP 2. Thereafter, the number of positive animals decreased to 15.4% at WPP 7 to 9. This decrease is significant. After infection this state remains until DPI 7, afterwards the number of positive animals rose to 90.9%.

Non-virus specific ADCC was shown by a few pigs between DPP 1 and DPP 5, indicating colostral transmission of Ab against non-viral vaccine constituents. Afterwards this activity disappeared.

It is noteworthy that no correlation existed between ADCC and the titres of the colostral neutralizing Ab. Animals with ND_{50} titres of 1:12 were positive in ADCC, others with titres of 1:195 were not, and vice versa. This differs from the situation after active immunization, especially after revaccination. Furthermore, the ability of colostral Ab to evoke ADCC was rather high shortly after birth but decreased with rising age. Colostral Ab are composed of IgG, IgA and

TABLE 6 Distribution of cell-mediated cytotoxicity in the body of pigs with colostral immunity (DPP1-12).

Test	Blood	Pharyng. + Ing. LNN	Mesenteric LNN	Spleen	Liver	Thymus
SCC	13%	13%	0%	30%	43%	5%
ADV-SCC	13%	9%	0%	0%	57%	0%
ADV-ADCC	65%	0%	0%	22%	43%	0%
ADV-LYST	0%	0%	0%	8%	0%	0%

IgM. From these only IgG react in ADCC, at least in our test system. It might have been that IgA and IgM had hindered the adsorption of IgG on the effector cells. However, during the first days after birth, when ADCC was present, the IgG/IgM/IgA ratio is 7:1:5, whereas on DPP 30 it is 12:1:1 (Jensen and Pedersen, 1979). Therefore, it is more likely that the colostral Ab have lost their cell affinity in the course of time.

ADV-LYST with WBC could not be detected before infection and during the first 4 days afterwards. It appeared from DPI 7 onward.

Colostral neutralizing Ab were present from DPP 1 to WPP 9. After infection active Ab production was first detected on DPI 7. Until this time, the ND_{50} values after infection apparently reflected colostral Ab.

Apart from the presence of colostral Ab the immunological response of colostral immune pigs after infection was very similar to that of non-immune infected pigs, which was shown before. Nevertheless, all the pigs with colostral Ab were protected against clinical AD . They only had elevated temperatures, which in some cases rose up to 40.6°C. One can conclude that colostral Ab were probably predominantly responsible for protection.

The 23 piglets which were examined between DPI 1 and DPI 12 were killed at these times to investigate the cytotoxic activity in different organs. Table 6 shows this distribution. It can be seen that SCC was predominant in spleen and liver, ADV-SCC in liver, and ADV-ADCC in blood and liver. ADV-LYST was only found in 2 cases with spleen cells and, as it will be shown later, with WBC too. Since these animals did not have contact with ADV it can be assumed that ADV-sensitized lymphocytes or ADV-sensitizing mediators had probably been transmitted via colostrum.

Vaccination of pigs having colostral Ab is a problem because active Ab production is inhibited by the colostral Ab and protection is not optimal. Therefore, the immunological behaviour of pigs with colostral Ab after vaccination and

TABLE 7 Immunological behaviour of pigs with colostral immunity after vaccination and infection.

		WPP		WPV		DPI		
		3	6	2	4-6	2	7	14
SCC		0/18 0%	1/5	8/12 66.7%	8/18 44.4%	10/18 55.6%	15/18 83.3%	7/18 38.9%
ADV-SCC		3/18 16.7%	0/5	8/12 66.7%	10/18 55.6%	8/18 44.4%	17/18 94.4%	16/18 88.9%
ADV-ADCC		0/18 0%	2/5	2/12 16.7%	4/18 22.2%	10/18 55.6%	5/18 27.8%	14/18 77.8%
ADV-LYST		2/18 11.1%	0/5	5/12 41.7%	1/11 9.1%	5/11 45.5%	9/11 81.8%	13/18 72.2%
ND_{50}*	a)	247		84	37	60	424	904
	b)	868	61	47	60	69	276	1826
Symptoms							Fever	

* Thirteen pigs (a) vaccinated at WPP 3, five pigs (b) vaccinated at WPP 6
Underlined significant ($p \leq 0.1$) with regard to WPP 3

after infection is of interest.

In Table 7 the results of 2 groups are summarized. The first group of 13 animals was vaccinated with an inactivated vaccine at WPP 3 and infected 4 to 6 weeks later. A second group of 5 animals was vaccinated at WPP 6 and infected 4 weeks later. Since, apart from the colostral ND_{50} values, the results of both groups were very similar it is allowed to compile the results.

SCC and ADV-SCC were only detected in a few animals before vaccination. After vaccination SCC as well as ADV-SCC significantly increased. Thereby, it cannot be decided, whether this was due to vaccination or due to the rising age of the animals. After infection the two cytotoxic activities further increased.

ADV-ADCC was not present at WPP 3, despite of high ND_{50} titres. After vaccination some animals became positive. After infection ADV-ADCC was significantly enhanced. The fact that not all of the animals showed significant ADV-ADCC was due to very high ADV-SCC cytotoxicity indices, which could not be significantly exceeded by the cytotoxic indices of ADCC.

ADV-LYST was detected with 2 animals at WPP 3, indicating colostrally induced sensitization of the lymphocytes. No significant indrease occurred after vaccination, but after infection the number of positive animals rose from DPI 7 onward.

The titres of colostral Ab were in the mean 1:420 at WPP 3 and 1:61 at WPP 6. When vaccination was done at 3 WPP, the Ab titres decreased afterwards down to 1:37. This means that no Ab production took place and the ND_{50} values found were those of colostral Ab. When vaccination was done at 6 WPP, the Ab titres remained rather constant what may be caused by limited Ab production. However, the number of animals tested was too small to allow a definite statement. After infection no significant booster effect was found on DPI 2, but it was present from DPI 7 onward.

In the last experiment we investigated the influence of revaccination on colostral immune pigs. The animals were first vaccinated when 3 weeks old, revaccinated 3 weeks later

TABLE 8 Immunological behaviour of pigs with colostral immunity vaccinated at 3 WPP, revaccinated at 6 WPP and infected 25 days later

	WPP	WPV	DPRV		DPI		
	3	3	12	25	2	7	14
SCC	1/7	1/7	4/7	4/7	0/7	5/7	2/7
ADV-SCC	5/7	1/7	3/7	3/7	5/7	7/7	7/7
ADV-ADCC	0/7	3/7	6/7	6/7	3/7	0/7*	7/7
ADV-LYST	0/7	3/7	5/7	1/7	2/7	7/7	5/7
ND_{50}	904	68	134	111	121	293	3382
Symptoms						Fever	

* Only lymphocyte fraction tested
Underlined significant in comparison to WPP 3

and infected 25 days afterwards. The results are given in Table 8.

SCC and especially ADV-SCC were found with a part of the animals at WPP 3. The first vaccination did not alter the reactivity of the animals, but after revaccination an insignificant enhancement of SCC was seen. Infection evoked not significant enhancement of SCC, but the number of ADV-SCC positive animals increased.

None of the animals showed ADV-ADCC on WPP 3, despite of high titres on neutralizing Ab. After the first vaccination positive animals were present and their number increased significantly after revaccination. After infection, no pig was positive on DPI 7, however, in this case we used purified lymphocytes for ADCC and thus the majority of reactive cells (monocytes and neutrophils) were not present.

ADV-LYST was not detected at WPP 3 but it occurred after vaccination and revaccination with a part of the animals and it was enhanced after infection on DPI 7.

The first vaccination did not evoke Ab production since the titres of colostral Ab fall from 1:904 before vaccination to 1:68 at WPV 3. Revaccination evoked in some of the animals a slight booster effect and on account of this the mean ND_{50} values rose to 1:134 on DPRV 12. On account of the half-life time of neutralizing ADV-Ab, which is about 10 days (Wittmann and Jakubik, 1979), one can assume that the level of the colostral Ab still present would have been in the mean about 1:34, however, the real ND_{50} value was 1:111. This indicates active Ab-production. After infection a moderate booster effect was found on DPI 7 and a very strong one on DPI 14.

Thus, revaccination could obviously overcome the inhibitory effect of colostral Ab. This result coincidents with earlier investigations (Wittmann, 1983).

All the vaccinated and revaccinated animals were protected against clinical AD when infected. They only showed fever of different intensity but no significant differences could be found between the 3 groups. However, differences existed with regard to the duration of nasal virus excretion.

This declined in succession non-vaccinated → vaccinated → revaccinated animals, but no group-significant differences in virus titres could be detected.

CONCLUSIONS

What general conlusions can be drawn from the results of the experiments? Since the animals did not show an uniform reactivity, but great individual variations, the number of animals used was too small to reflect the real situation. However, some tendencies can be noticed nevertheless.

At infection the degree of spontaneous cell-mediated cytotoxicity against ADV infected cells with and without interferon participation may be of importance for the further course of infection and of clinical disease. This corresponds with results found with HSV and murine cytomegalo virus (Ching and Lopez, 1979; Quinnan et al., 1982). Nothing is known at present about the role of CTL in the early phase of infection. However, with other herpes virus infections about one week elapses until CTL are demonstrable (Quinnan et al., 1978, 1980; Rouse and Babiuk, 1977). Ab production does not occur until the initiation of pathologic processes. Therefore direct Ab/virus interaction, complement-dependent cytolytic effects of Ab (Martin et al., 1983), ADCC and T-cell cytotoxicity might only be of influence in later phases of infection and in recovery.

In vaccinated pigs neutralizing Ab seem to be the main factor of protection against ADV infection. They can act as described before. However, revaccination is necessary to evoke an optimal immunological Ab-dependent response. Besides, spontaneous cell-mediated cytotoxicity may limit local ADV multiplication. T-cell participation remains still open. The occurrence of fever without clinical signs of AD in vaccinated pigs might be an expression of immunological cytotoxic events in the body. However, protection against infection is apparently not only a matter of the immune state, but it is also influenced by different forms of the pathogenesis of ADV infection. For example, vaccinated cattle

with high serum Ab titres were not protected against ADV (Wittmann et al., 1983). Thus the virus must have multiplied on places and spread on ways which were not accessible to immune defense, e.g. neural tissue. The same is true when the virus is spread in lymphocytes and in macrophages.

In colostral immune pigs the colostral Ab and their different reactions may be of main importance for protection against ADV infection, thereby ADCC apparently plays a secondary role. Besides sponataneous cell-mediated cytotoxicity may act immediately after infection. The inhibitory effect of colostral Ab on active Ab production after vaccination can be overcome by revaccination, and various Ab-dependent immunological events take place.

However, these statements are theoretical considerations and, as with other herpes viruses (Rouse and Babiuk, 1978), nobody knows what really happens in the infected animal, especially with regard to cell-mediated immunity, because we have predominantly investigated the cytotoxic response of peripheral blood cells, and not the cytotoxic events in the organs. Besides, we have completely disregarded the local immunological responses taking place at the places of virus entry.

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SOME ASPECTS OF BHV 4 INFECTION IN CATTLE

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ABSTRACT

The authors describe some virological, immunological and clinical aspects of BHV 4 infection in cattle under field conditions and after experimental inoculation. The virus was isolated in several cases of post parturient metritis up to several weeks after the onset of symptoms. The virus was evidenced in all the examined lymph nodes. With the direct immunofluorescence test only few isolated cells were fluorescing in uterine mucosa and lymph nodes slides. Important fluorescent plages were not demonstrated.

After recovery of the animals, no neutralizing antibodies were evidenced. But with the indirect immunofluorescence test and the ELISA test, a clear seroconversion was demonstrated.

The chronic nature of this affection was explained by the absence of neutralizing antibodies in most of the affected animals. The associated symptoms, observed in the affected herds (diarrhoea, mortinatality, respiratory problems in cows and calves, mastitis) were obviously not due to the direct action of the virus. This Herpesvirus could play an immunodepressive role favouring the development of secondary affections.

VIROLOGICAL AND SEROLOGICAL STUDY

During the winter 1982-1983, a cytopathogenic virus was isolated from the lochia of more than 20 cows affected with chronic metritis. This metritis appeared from the fifth postparturient day on, and was characterized by a slow evolution and repeated breeding problems. The vaginal discharge was not very abundant, sanguinolent till brownish, and continued for several months in some cases.

In 4 farms the metritis was accompanied by a painful peritonitis, resulting in the death of the animal or emergency slaughter in most of the cases.

Viral isolations were chiefly obtained from the mixture of uterine discharge and, in two cases, from the peritoneal liquid taken resp. 3 weeks and 3 months after the onset of symptoms.

The isolated viruses presented all the characteristics of the Herpes viruses : type A Cowdry intranuclear inclusions, sensitivity to chloroform

and to acid pH, morphology in electron microscope examination, etc... These viruses were not recognized in the direct immunofluorescence test nor neutralized by our anti-IBR (infectious bovine rhinotracheitis), Aujeszky and BHM (Bovine Herpes Mammitis) antisera. At the other hand our anti-Movar (Mohanty et al., 1971) and DN 599 (Potgieter and Mare, 1974) antisera crossreacted with these viruses (see table 1).

The cytopathogenic effect (CPE) appeared slowly after 1 to 2 weeks The first CPE appeared often only after 2 passages, whereas the presence of virus in the cell culture was very rapidly (2 to 3 days) detected with the direct immunofluorescence test. The following passages were faster (3 - 4 days).

No typical fluorescent lesions were detected with the direct immunofluoresence (DIF) test on ultrathin sections of genital organs. Only few disseminated positive cells were found in the affected mucosae. This absence of a clear cut infection localisation suggested a type of infection different from the type usually induced by other viruses.

Only two out of 66 recovered animals showed seroneutralizing antibodies, and the observed titers were very low (1/2 and 1/4), though the indirect immunofluorescence (IIF) and ELISA titers were very high. The reduction of infected cells - test confirmed the lack of neutralization of infectious particles by the convalescent sera (see table 2).

This absence of neutralization could explain the chronicity of the infection, and the presence of infectious particles in samples till 3 months after the onset of clinical symptoms. These findings correspond with the statements made by other authors concerning BHV 4 infection in cattle (Osorio and Reed, 1983; Mohanty et al., 1971; Parks and Hendrick, 1973).

EXPERIMENTAL INOCULATION STUDY

The first isolate (LVR 140) was inoculated intravenously in 6 cows in late pregnancy. Five cows were slaughtered one month after parturition, one cow died one week after calving.

The following conclusions were drawn from this experimental inoculation :

1. The 6 cows presented a metritis with vaginal discharge starting a few days after parturition. A LVR 140 virus was isolated. The inoculation was performed in some cases more than one month before parturition.

2. The same virus was isolated from the uterine mucosa of the animals slaughtered one month after parturition. Nevertheless, no characteristic lesions were found with the DIF test, besides a few disseminated lesions.
3. In all the examined lymphnodes (retromammary, prescapulary, precruraly, iliacal and mediastinal) and the liver (in 3 out of 6 cows), LVR 140 virus was isolated. Here also disseminated fluorescent cells were detected with the DIF test.
4. The evolution of IIF antibodies was situated on two levels as shown in fig.1. No neutralizing antibodies were detected in the sera obtained at slaughter. The viral reisolation and the evolution of antibodies show that no immunization was developed after inoculation and that the stress of delivery induced the genital pathology.
5. No IIF nor ELISA antibodes were detected in the precolostral blood samples of the newborn calves. The virus seems thus not to pass the placental barrier.
6. One cow presented nasal discharge after parturition and another cow died one week after delivery without showing clear morphological lesions at autopsy.

FIELD STUDIES

When studying the anamneses obtained in 21 affected farms (see table 3) it became obvious that the postparturient metritis was not the only observed symptom. This genital pathology was often accompanied by other troubles affecting most of the organic systems.

In 11 farms nasal discharge and coughing was noted in affected cows and their calves. Smith et al., 1972 and Bartha et al., 1976 described the isolations of BHV 4 virus in similar cases. Neonatal diarrhoea was observed in 11 farms. Fecal material of calves form 4 farms contained important quantities of Cryptosporidium oocysts whereas in 3 other farms no valid diagnosis was established.

In 8 farms mammitis symptoms were stated, characterized by an induration of the mammary gland and a significant enhancement of the cell number. Bacteriological examination revealed only ordinary bacteria and high numbers of yeast cells.

Frequent mortinatilities without apparent causes were observed

in 7 farms. No disease symptoms, explaining these sudden deaths, were found at autopsy.

Although no LVR 140 virus was found, if cherched, in other than the genital organs, it seems not much probable in view of their frequent apparence that these symptoms were due to chance.

In the numerous samples (fecal material, milk, nasal mucous, different samples at autopsy) only microbial or ordinary parasites (streptococcus, Cryptosporidium, yeast cells), thus few or non pathogenic agents, were detected.

One may consequently suppose that this Herpesvirus play an immunodepressive role or that the virus profits on a decreased general resistance for its development in a herd (Van Opdenbosch et al., 1984).

However, the experimental inoculations seem to be in favour of the first hypothesis although we did not succeed in inducing clearly an immunodepressive state in the animals kept in isolated stables.

DISCUSSION

The mean symptom of this BHV 4 associated symptomatology is the chronic metritis with or without peritonitis. The chronic nature of the affection was explained by the absence of neutralizing antibodies. This allows a persistent virus excretion as appeared from the virus isolation from peritoneal liquid 3 weeks and 3 months after the onset of symptoms. The virus could be isolated only from lochia, peritoneal liquid and lymphoid tissues.

From other samples (fecal material, nasal discharge, milk samples) only ordinary bacteria and parasites were isolated (Cryptosporidium, Streptococcus and yeast cells).

No fluorescent lesions were evidenced with the DIF test. So the replication place of the virus was not determined. The similarity of this virus with MOVAR 33/63 (Mohanty et al., 1971), DN 599 (Potgieter and Mare, 1974) and the virus isolated by Thiry (Thiry et al., 1981) was demonstrated. The lymphoid-associated persistent infection may have marked implications in immunological disorders in cattle (Osorio and Reed, 1983). This could be an explanation of the great diversity of symptoms that we observed.

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TABLE 1 Comparison between bovine Herpesvirus strains

Strains	Origine	Serum LVR 140			Ser.IBR	Ser.AUJ.
		D I F		S N	DIF	DIF
		A*	B**			
Movar 33/63	Bartha	-	+	+	-	-
DN 599	Mohanty	-	+	+	-	-
X	Thiry	-	+	+	-	-
CV 142/72	NIVR	-	+	+	-	-
LVR 140	NIVR	-	+	+	-	-
IBR3760	NIVR	-	-	-	+	-
Aujeszky	NIVR	-	-	-	-	+
BHM-TVA	Gaskell	-	-	-	-	-

* Calf serum before immunization

** Calf serum after immunization

NIVR : National Institute for Veterinary Research

TABLE 2 Research of LVR 140 antibodies in the affected herds

Farms	I I F test		ELISA	SN
		Mean titer		
2	4/4*	5120	4/4	1/4 (1/2)**
3	2/2	5120	1/1	0/1
5	9/10	5120	10/10	0/5
6	2/2	1280	2/2	0/2
7	2/2	5120	2/2	0/2
8	5/5	5120	5/5	0/5
9	6/6	1280	6/6	1/6 (1/4)
10	15/15	5120	15/15	0/9
11	20/20	1280	20/20	0/20

* Number of positives per number examined

()** Titer (50 $CCID_{50\%}$)

TABLE 3 Symptoms in 21 problem herds in which the LVR 140 virus has been diagnosed (isolation + serology)

	SYMPTOMS OBSERVED				
Herds	Metritis	Mastitis	Resp.Dis.*	Neo.Diar.**	Sudden death
1	+	+	+	+	+
2	+				
3	+				
4	+	+	+	+	+
5	+		+	+	+
6	+	+		+	
7	+		+	+	
8	+		+		
9	+	+			
10	+	+	+	+	
11	+	+	+	+	
12	+	+	+	+	+
13	+		+		+
14	+				
15	+			+	+
16	+		+		+
17	+				
18	+		+	+	
19	+				
20	+	+			
21	+			+	
Total	21	8	11	11	7

* Respiratory disorders

** Neonatal diarrhoea

FIGURE 1 Evolution of IIF anti-BHV 4 antibodies around parturition

STUDIES OF VIRULENCE GENES OF HERPES SIMPLEX VIRUS (HSV-1)

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ABSTRACT

Studies in mice and in the animal model system tree shrew suggest that the gene functions responsible for the virulence of HSV-1 in vivo are located between the viral genome coordinates of 0.71 and 0.83 map units. Herpes simplex virus (HSV) type 1 strain HFEM whose genome contains a deletion of about 4 kilobasepairs (kbp) between 0.7 and 0.8 map units is pathogenic in the tree shrew when the animals are inoculated intravenously, intraperitoneally and/or subcutaneously. Similar results were found using Balb/c mice. Studies of the state of viral latency in animals infected with HSV-1 strain HFEM revealed that this strain was unable to colonize the ganglia of tree shrews. In contrast infectious virus could be recovered only from the spleens of latently infected tree shrews. Thus, this system offers new opportunities for investigating the gene functions responsible for the virulence of HSV-1. Marker rescue experiments were performed by the cotransfection technique using native DNA of HSV-1 strain HFEM and the Bam HI DNA fragment B which was derived from the pathogenic HSV-1 strain F and cloned in a bacterial vector. A variety of intratypic recombinants was established in which the deleted genome region of HSV-1 strain HFEM was replaced. The pathogenicity of these recombinants was examined in vivo. Two out of nine recombinants caused generalized and lethal herpesvirus infection in the tree shrew.

The exact map position of the deletion in the genome of HSV-1 strain HFEM was determined using a recombinant plasmid constructed by insertion of the Bam HI DNA fragment B of the HSV-1 strains HFEM, F, and different recombinant viruses in the plasmid vector pAT153. The analysis of the insert of these recombinant plasmids using a variety of restriction enzymes and Southern blot hybridization revealed that the Hpa I DNA fragment P of the intact viral genome of HSV-1 strain F is not present in the genome of HSV-1 strain HFEM indicating that the deletion in HSV-1 strain HFEM corresponds to the Hpa I DNA fragment P spanning the map coordinates 0.762 to 0.790 of the genome of HSV-1 strain F. The size of the deletion was found to be 4.1 kbp.

INTRODUCTION

The investigation of the pathophysiological mechanism of herpes simplex virus (HSV) infection is of special interest, since it focusses attention on the determination of the gene function responsible for tropism and virulence of HSV. Different animals, especially rodents, have been used in the last

decade to study tropism, pathogenicity and latency of HSV. The pathogenicity of the different HSV types 1 and 2 has been investigated in the animal model system tree shrew (Darai et al. 1978, 1980, 1982, 1983, 1983a; Scholz et al. 1983; Darai & Scholz, 1984). These studies revealed that HSV-1 and HSV-2 are highly pathogenic in juvenile and adult tupaias. An infectious dose of less than 1.0×10^3PFU led to 100% lethality in juvenile animals. The clinical picture of the HSV infection was manifested as a state of herpetic hepatitis when the animals were inoculated intravenously, intraperitoneally and subcutaneously. The latent HSV was reactivated from the ganglia of those adult animals which survived an acute HSV infection and were sacrificed several months and/or years after inoculation. The genomic alterations at 0.1-0.22, 0.73-0.82 and 0.94-1.0 map units of the HSV-1 genome were found to be associated with the avirulence and change of organotropism for latent HSV from the ganglia of the central nervous system (CNS) to the spleen in the reticular endothelial system (RES) in the tree shrew (Scholz et al. 1983; Darai & Scholz 1984). Thompson et al. (1983) recently reported that HSV-1 DNA region from 0.71-0.83 map units was especially associated with the function responsible for neurovirulence when tested in mice. Therefore it was of importance to prove the role of this gene region.
The suspected gene region of the nonpathogenic HSV-1 can now be studied more closely by replacing it with the corresponding intact cloned DNA fragments of a pathogenic HSV strain using DNA cotransfection experiments and this is the subject of this report.

MATERIAL AND METHODS

The experimental approach for this investigation (e.g. virus, animals, cocultivation, preparation of viral DNA, molecular cloning, transfection procedure, restriction enzyme analysis, Southern blot hybridization and nick translation) was carried out as described previously (Darai et al. 1978, 1980, 1982, 1982a, 1983, 1983a, 1984; Rösen et al. 1984; Rösen & Darai 1985).

RESULTS

Following a primary infection presenting with different clinical pictures HSV usually persists in a latent state and remains lifelong in the animal. Spontaneously reactivated latent virus can result in other clinical diseases. The mechanism of this complex process is not completely clear. To understand the mechanism many detailed biological and molecular-biological investigations are necessary. A first step for achieving this goal is to determine the gene functions and the corresponding gene regions responsible for the biological properties of HSV in vivo, such as viral tropism, virulence and latency. Recently the genome regions at 0.72-0.83 map units (Scholz et al. 1983) and/or 0.71-0.83 map units (Thompson et al. 1983) of HSV-1 were suspected to be involved in the process of pathogenicity and neurovirulence. The following strategy was chosen to investigate these observations in more detail.

Experimental approach

HSV-1 strain HFEM with a deletion of 4 kbp between the coordinates at 0.7 and 0.8 map units of viral genome, which is known to be apathogenic in mice (Halliburton, personal communication), was used for the experimental infection of tree shrews. For demonstrating that the deleted region in the genome of HSV-1 strain HFEM is indeed located in the Bam HI DNA fragment B of the viral DNA it was analysed using a variety of restriction endonucleases and the resulting cleavage patterns were compared to the intact viral genome of HSV-1 strain F. It was found that the deleted Bam HI DNA fragment B of HSV-1 strain HFEM is resistent to restriction endonucleases Eco RI, Hind III, Bgl II, Pvu II, and Xba I. The homology of the DNA sequences of the deleted Bam HI DNA fragment B of HSV-1 strain HFEM to the intact Bam HI DNA fragment B of HSV-1 strain F was conformed using Southern blot hybridization test. ^{32}P-labelled DNA of the recombinant plasmid pHSHF-B-B harbouring the Bam HI DNA fragment B of HSV-1 strain HFEM was hybridised to Bam HI digested DNAs of HSV-1 strain F, HSV-1 strain HFEM, recombi-

nant plasmid pHSF-B-B harbouring Bam HI DNA fragment B of HSV-1 strain F, and pHSHF-B-B. The results of this analysis are shown in Figure 1. Positive hybridization was found to the DNA sequences of the Bam HI DNA fragments B and E of HSV-1 strain F (Figure 1B, lane 3), to the corresponding Bam HI DNA fragments B and E of HSV-1 strain HFEM (lane 5), and to the individual recombinant plasmids pHSF-B-B (lane 4) and pHSHF-B-B (lane 6).

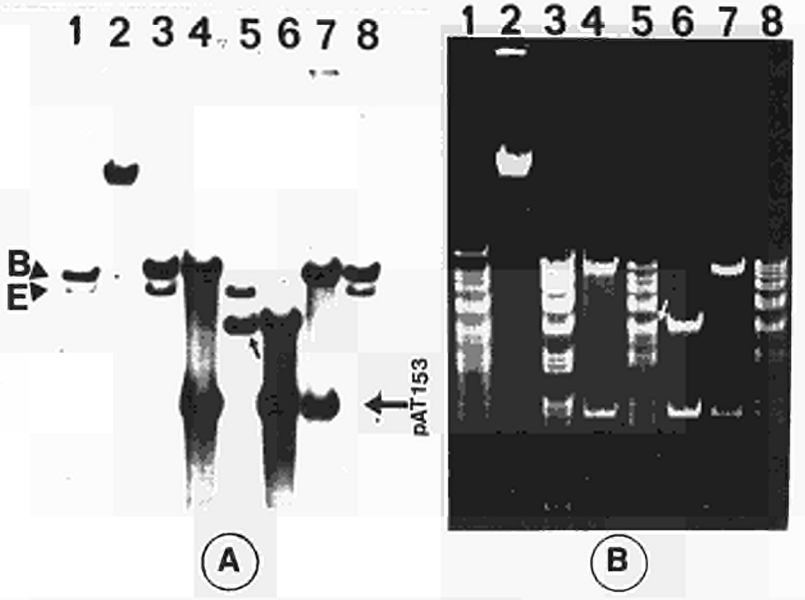

Fig.1 Characterization and identification of the DNA of recombinant plasmids using restriction endonuclease analysis and Southern blot hybridization. A composition of DNA fragments of recombinant plasmids in comparison to the DNA fragment of parental viruses after cleavage with restriction enzyme Bam HI. The DNA fragments were separated on agarose slab gel (0.8%), and the electrophoresis was performed at constant voltage (75 V), at 4°C, for 20 hours. Ethidium bromide staining and photographed under UV light (B). Autoradiogram of the same gel (A) after Southern blot hybridization using ^{32}P-nicktranslated pHSHF-B-B plasmid DNA. Lanes: 1, HSV-1 strain 17 served as internal marker; 2, HSV-1 strain F undigested; 3, HSV-1 strain F; 4, recombinant plasmid pHSF-B-B; 5, HSV-1 strain HFEM; 6, recombinant plasmid pHSHF-B-B; 7, recombinant plasmid pHSR19-B-B; and 8, recombinant virus strain HSV-R-HFehx-C19.

Arrows mark the position of the deleted Bam HI DNA fragment of HSV-1 strain HFEM.

It is well documented that the tree shrew is highly susceptible to wild-type HSV-1 and 2 which causes 100% lethality (Table 1) in juvenile animals by generalized viral infection and predominantly herpetic hepatitis (Darai et al. 1978, 1980).

TABLE 1 Pathogenicity of herpes simplex virus 1 and 2 (wild type) in Tupaia belangeri (tree shrew).

Wild type virus	Route of infection	Lethality juvenile	Lethality adult	Clinical picture of illness
HSV-1	s.c., i.m., i.p., i.v.	100 %	80.7 %	Generalized Herpes Virus Infection -HERPETIC HEPATITIS-
	p.o.			Necrosis of Stomach, Intestine and Adrenal Gland
HSV-2	s.c., i.m., i.p., i.v.	100 %	72.3 %	Generalized Herpes Virus Infection -HERPETIC HEPATITIS-
	p.o.			Necrosis of Stomach, Intestine and Adrenal Gland

s.c.: subcutaneously, i.m.: intramuscularly, i.p.: intraperitoneally, i.v.: intraveneously, p.o.: per os.

If HSV-1 strain HFEM was not able to induce generalized herpesvirus infection in the tree shrew this system could be applied to the second step of this investigation, in which the deletion in the altered gene regions of the nonpathogenic HSV-1 strain HFEM could be repaired by replacing it with the corresponding intact cloned DNA fragment of a pathogenic HSV-1 strain using DNA cotransfection experiments which allow the generation of intratypic recombinants. One can assume that genetic information coded on the DNA sequence of the Bam HI DNA fragment B from HSV-1 strain F which is pathogenic for the juvenile tupaia could be transduced to the apathogenic HSV-1 strain HFEM using this experimental approach (Table 2). Therefore the recombinants generated should acquire new properties concerning their pheno- and/or genotype and could be virulent in vivo for juvenile tupaias.

TABLE 2 Transduction of virulence in herpes simplex virus type 1 from a pathogenic to an apathogenic strain by a cloned viral DNA fragment.

PATHOGENIC STRAIN:	HSV-1 strain F
CLONED VIRAL DNA:	Bam HI DNA fragment B from HSV-1 F
APATHOGENIC STRAIN:	HSV-1 strain HFEM with a deletion* between 0.7 and 0.8 map units of viral genome.
TRANSDUCTION METHOD:	DNA-cotransfection on BHK-1 cells
RECOMBINATION EVENT:	Homologous, intratypic
TEST ANIMAL:	tree shrew

* The size of the deletion was found to be 4.1 kilobase-pairs corresponding to 2.7 megadalton; spanning the co-ordinates 0.762 to 0.790 of the intact HSV-1 genome (Rösen & Darai 1985).

Pathogenicity and state of viral latency of HSV-1 strain HFEM in tree shrew and mice.

The susceptibility of juvenile tree shrews to HSV-1 strain HFEM was investigated.The animals were inoculated by different routes (i.v., s.c., i.p., and i.c. with 1×10^7PFU / animal). The results of this study are shown in Table 3, part 1. It was found that HSV-1 strain HFEM was not pathogenic in the tree shrew when the animals were inoculated intravenously, subcutaneously and/or intraperitoneally. No clinical picture of illness was observed in these animals. As shown in earlier reports different strains of other HSV types 1 and 2 with intact genomes are able to kill juvenile tree shrews within a few days (1×10^3PFU / animal) with the clinical picture of generalized herpetic hepatitis (Darai et al. 1978, 1980). In contrast it was found that after direct intracranial administration of HSV-1 strain HFEM the animals developed a focal encephalitis following an infection with 1×10^7PFU / animal. These animals showed the clinical picture of paresis and were sacrificed when moribund and autopsied immediately after death. The analysis of the different specimens from these animals for

TABLE 3 Sensitivity of juvenile tree shrews and Balb/c mice to infection with HSV-1 strain HFEM in comparison to the HSV-1 strain F.

Part	HSV-1	PFU of virus/animal	No. of animal dead/No. of animals inoculated	Time of death days p.i.	Titre of HSV (PFU/g*tissue) Brain	Liver	Blood	Virus recovered from ganglia	spleen
1(T)	HFEM	1.0×10^6 (s.c.)	0/2					No	Yes
		1.4×10^7 (i.p.)	0/4						
		2.5×10^7 (i.v.)	0/2						
		1.0×10^7 (i.c.)	2/2	4	1.5×10^3	<1	<1		
				5	4.0×10^2	<1	<1		
	HF-SP-1‡	1.8×10^7 (i.v.)	0/1					No	Yes
	HF-SP-2§	6.0×10^7 (i.p.)	0/1					No	Yes
	HF-SC+	$1\text{-}5\times10^7$ (i.v.)	0/10					No	Yes
	HF-Tr.++	5.0×10^7 (i.v.)	0/5					No	Yes
2(T)	F	5.0×10^2 (i.p.)	2/2	5	2.7×10^4	2.3×10^7	7.2×10^3		
				6	1.8×10^4	5.4×10^7	6.5×10^2		
3(M)	HFEM	1.0×10^7 (s.c.)	0/10					not done	
		1.0×10^7 (i.p.)	0/11						
		1.0×10^7 (i.c.)	9/10	3-9	52 to 3.2×10^6	<1	<1		
4(M)	F	1.0×10^6 (i.p.)	4/4	3-4					

* plaque forming units/gram tissue; the resulting titres of virus obtained from the plaque assay were normalised to 1.0 gram of the corresponding original tissue.

‡ virus isolated from the spleen of animal No.T13 which was infected with HSV-1 strain HFEM.
§ virus isolated from the spleen of animal T31 which was infected with HSV-1-T13-HFEM-SP1.
+ the animals were inoculated with a stock of ten different subclones of HSV-1-HFEM which were established from individual single plaques.
++ the animals were inoculated with five virus stocks of HSV-1-HFEM which were established from individual single plaques isolated after transfection of BHK-21 cell cultures with native DNA of HSV-1-HFEM.
T = Tupaia
M = Mouse

virus isolation and titration revealed only very low titers (4×10^2 to 1.5×10^3 PFU/g tissue) in the brain tissue of the intracranially infected animals (Table 3, part 1).

Detailed necropsy and histopathology showed that the only affected organ was the brain. The meninges were partially hemorrhagic and adhering to the skull only in the injection area. No specific alterations were found in other organs. The individual viruses isolated from the brain tissue of the animals were identified by analysis of the viral genomes. Comparisons of the DNAs of these isolates and the DNA of the original inoculum by restriction enzyme analysis revealed no changes in the fragmentation patterns.

For comparison, the data obtained in this study and the pattern of virus spread and viral progeny in tree shrew after generalized herpesvirus infection with HSV-1 strain F are presented in Table 3, part 2. To demonstrate the lack of pathogenicity of HSV-1 strain HFEM, additional experimental infections were performed using Balb/c mice. The results of these studies, as shown in Table 3, part 3 and 4 are completely similar to data obtained from tree shrew. Taken together, this indicates that HSV-1 strain HFEM with a deletion of 4 kbp of its genome located on the Bam HI DNA fragment B is neither pathogenic in tree shrew nor in Balb/c mice under these conditions.

The state of viral latency was studied in those tree shrews which survived an infection with HSV-1 strain HFEM. The animals were sacrificed at different times after infection and specimens of blood, purified leukocytes, brain, cervical, thoracolumbar and sacral segments of spinal cord, thymus, spleen, and kidney were screened for recovery of infectious viruses by the cocultivation technique using RC-37 cell cultures. Although a variety of specimens from the infected animals was used for recovery of latent virus, infectious HSV was recovered only from the spleen of the animals as shown in Table 3, indicating that the target organs responsible for the persistence of latent HSV-1 strain HFEM in the tree shrew was the spleen of the reticulo-endothelial system and not the ganglia of the central nervous system as known for wild-type HSV. The analysis of the genomes of the various viruses isolated from the spleen of the animals, which were picked individually from single plaques,

was performed. The DNAs of each isolate were cleaved with different restriction endonucleases and the resulting DNA fragments were separated by slab gel electrophoresis. A comparative analysis of the DNAs from these isolates and the DNA from the original inoculum by restriction enzyme analysis revealed no changes in the fragmentation patterns (Rösen et al. 1984). Since the biological properties of the viruses isolated from the spleen were of special interest, some of these strains were used to re-infect tree shrews. Juvenile tree shrews were inoculated as shown in Table 3, part 1, with such re-isolates under the same conditions as used for HSV-1 strain HFEM. As shown in Table 3, part 1, pathogenicity and latency of those isolates which were recovered from the spleens of tree shrews were similar to those of HSV-1 strain HFEM.
The results obtained from experimental infection in tree shrews with HSV-1 strain HFEM demonstrate clearly that this strain cannot induce generalized herpesvirus infection in the tree shrew. Therefore this system is the model of choice for further molecularbiological studies. Ultimately these studies should prove whether or not a correlation exists between the presence of the deletion located on the Bam HI DNA fragment B of the genome of this strain and the apathogenicity of this virus in tree shrew.

Generation of intratypic recombinants for replacing the deleted region.

Three pmol of cloned Bam HI DNA fragment B of HSV-1 strain F which were derived from recombinant plasmid pHSF-B-B were coprecipitated with 0.01 pmol native intact DNA from HSV-1 strain HFEM and used for transfection of BHK-21 cell cultures. When generalized cytopathic effects occurred the total cultures were frozen, thawed and sonicated and the cell free supernatant was used for single plaque isolation. The individual single plaques were screened using ^{32}P-labelled viral DNA and digestion with restriction endonucleases. As shown in Figure 2 one novel Bam HI DNA fragment appeared in the slab gel electrophoresis between the migration position of the

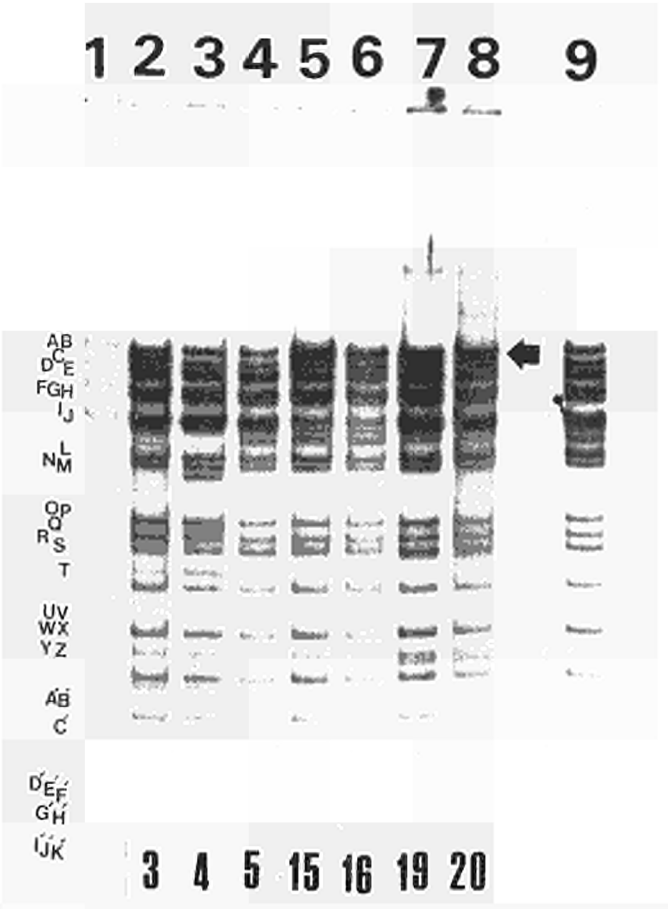

Fig.2 Autoradiograms of ^{32}P DNAs of different recombinant viruses in comparison to the parental virus HSV-1 strain HFEM (lane 9) and HSV-1 strain F (lane 1) from which the Bam HI DNA fragment B was derived and cloned in pAT153 and used for cotransfection experiments. The DNAs were cleaved with restriction endonuclease Bam HI. The letters indicate the position of the individual Bam HI DNA fragments of HSV-1 strain F (Mocarski & Roizman, 1982). The position of the deleted Bam HI DNA fragment B of HSV-1 strain HFEM is marked with an asterix. Lanes are given at the top of the picture and the number of the individual recombinant virus strains at the bottom of the picture. The big arrow marks the position of the novel Bam HI DNA fragment B in recombinants C3 (lane 2), C15 (lane 5), C16 (Lane 6), C19 (lane 7), and C20 (lane 8).

Bam HI DNA fragments A and C of HSV-1 strain HFEM DNA indicating that intratypic recombination events in this specific region of the genome of HSV-1 strain HFEM must have taken place. Twelve out of nineteen single plaque isolates showed this new fragment. The size of this newly developed DNA fragment varied and reached a maximum size of 10.2 kbp in some of the recombinants. Further detailed analyses using different restriction endonucleases were carried out to identify the new DNA fragment. It was found that the newly generated Bam HI DNA fragment was resistant to Hind III (Figure 3), Eco RI and Bgl II, and Xba I (data not shown).

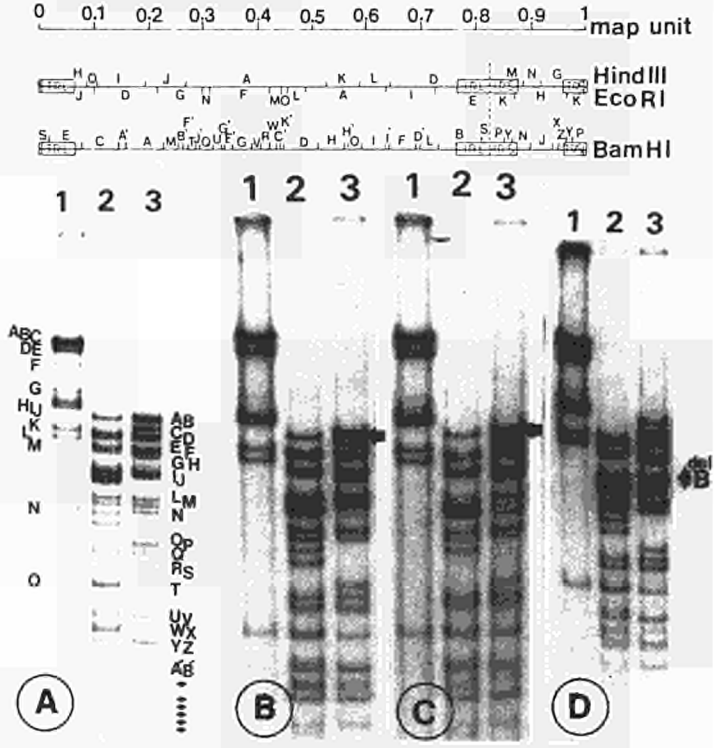

Fig.3 Autoradiograms of ^{32}P DNAs of two recombinants C19 (panel B) and C15 (panel C), and HSV-1 strain F (panel A) and HSV-1 strain HFEM (panel D). The DNAs were digested with Hind III (each lane 1), Bam HI (each lane 3), and double digestion with Bam HI and Hind III (each lane 2). The arrows mark the position of the novel generated Bam HI DNA fragment B.

In contrast, Hpa I (Figure 4) cuts this novel generated Bam HI DNA fragment indicating that this DNA fragment originates from the Bam HI DNA fragment B. The different size of the newly generated DNA fragment in different isolates is due to the slightly variable length of the DNA sequences which recombined from the cloned Bam HI DNA fragment B of HSV-1 strain F into the corresponding genome region of HSV-1 strain HFEM.

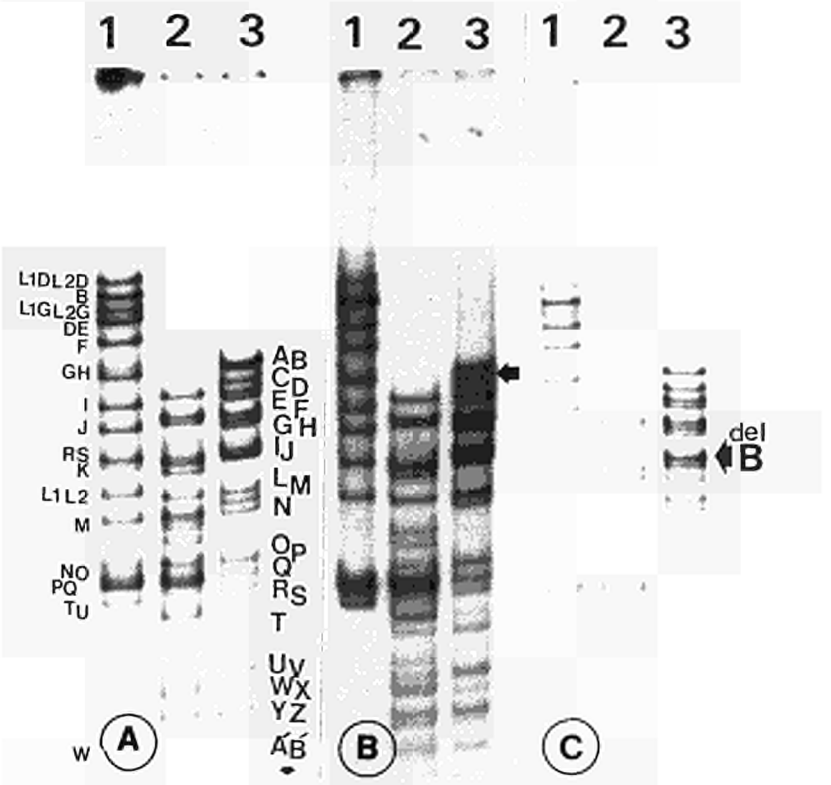

Fig.4 Autoradiograms of ^{32}P DNAs of recombinant virus HSV-R-HFehx-C19 (panel B), HSV-1 strain F (panel A), and HSV-1 strain HFEM (panel C). The DNA samples were digested with restriction endonucleases Hpa I (each lane 1), Bam HI (each lane 3), and double digestion with Hpa I and Bam HI (each lane 2). The small arrow marks the position of the novel generated Bam HI DNA fragment B.

Characterization of recombinants:
Biological properties

The cytopathic effect (CPE), the formation of syncytia and the size of plaques produced by individually isolated recombinants were studied in comparison to the parental HSV-1 strain HFEM and HSV-1 strain F from which the Bam HI DNA fragment B was cloned. Some of the recombinants induced only round cell CPE and others small syncytia on RC-37 cell cultures. The biological properties of the isolated recombinants are summarized in Table 4.

TABLE 4 Properties of intratypic recombinants of HSV-1 strain HFEM harbouring DNA sequences of Bam HI DNA fragment B from HSV-1 strain F

Number of recombinant HSV-R-HFehx-C:	Genotype	Phenotype*			Pathogenicity in juvenile tree shrew
	size of new Bam HI DNA fragment B Kbp	CPE	plaque size mm Ø	virus yield PFU x ml^{-1}**	
3	10.1	RC	0.5-0.8	1.87×10^8	apathogenic
15	10.2	SS	0.1-0.2	1.31×10^7	
16	10.2	RC	0.1-0.2	3.33×10^7	
19	10.1	SS	0.8-1.0	1.91×10^8	PATHOGENIC***
20	9.6	RC	0.1-0.3	ND	
21	10.1	SS	1.5-2.0	2.06×10^7	apathogenic
25	10.2	SS	1.5-2.0	5.35×10^6	
26	10.2	RC	0.5-0.8	3.28×10^7	PATHOGENIC***
34	10.1	RC	0.1-0.2	2.75×10^7	apathogenic
39	9.9	RC	0.4-0.5	1.41×10^7	
HSV-1 HFEM	6.5	LS	1.5-2.5	1.72×10^7	apathogenic
HSV-1 F	10.5	RC	0.6-0.8	7.81×10^7	pathogenic

* VIRUS WAS PROPAGATED IN MONKEY KIDNEY CELL CULTURE (RC-37)
** RC-37 CELL CULTURES WERE INFECTED WITH A MOI OF 0.01 PFU/CELL OF EACH VIRUS STRAIN AND INCUBATED AT 37°C FOR 24 HOURS.
*** 100% LETHALITY IN JUVENILE TREE SHREW
ND = NOT DONE
RC = ROUND CELL CPE, SS = SMALL SYNCYTIAL PLAQUES, LS = LARGE SYNCYTIAL PLAQUES
CPE = CYTOPATHIC EFFECT

Pathogenicity

To answer the cardinal question in this experiment whether or not the replacement of the deleted genome region of the apathogenic HSV-1 strain HFEM with the corresponding cloned DNA fragment from the pathogenic HSV-1 strain F can lead to

the generation of pathogenic HSV recombinants, the following experiments were done. The recombinant viruses HSV-R-HFehx-C3, 15, 16, 19, 21, 25, 26, 34, and 39 were used for testing their virulence in the tree shrew. Juvenile tree shrews were inoculated intraperitoneally with individual HSV recombinants by administration of 1×10^6 PFU / animal. Among the recombinant virus strains tested, the recombinants HSV-HFehx-C19 and C26 were found to be pathogenic for tree shrews. The animals developed the clinical picture of generalized herpesvirus infection and died five days after inoculation. Histopathological studies of different organs revealed a generalized herpesvirus infection. The liver tissue showed several parenchymal changes with foci of hemorrhagic necrosis, a state of typical herpetic hepatitis. The electron microscopy showed herpesvirus nucleocapsids in different stages of maturation mainly within the nuclear portions of liver cells. For the determination of new viral progeny and virus spread a variety of specimens including blood, liver, spleen, kidney, adrenal gland and brain were taken from the animals and tested by plaque assay. The results of these assays, which are shown in Table 5, demonstrate clearly the state of generalized herpesvirus infection in juvenile and adult tree shrews after intraperitoneal administration of recombinant viruses HSV-R-HFehx-C19 and C26. Individual viruses isolated from different tissues were identified by analysis of the viral genomes. A comparison of the DNAs from these isolates and the DNA of the originally inoculated virus by different restriction enzyme analyses revealed no changes in the fragmentation patterns, especially with respect to the novel replaced Bam HI DNA fragment B, thus indicating the genetic stability of the genomic arrangement of recombinant viruses HSV-R-HFehx-C19 and C26 after in vivo passage.
The susceptibility of juvenile and adult tree shrews to infection with recombinant virus HSV-R-HFehx-C19 was investigated in more detail. The animals were inoculated with an infection dose of 1×10^2 to 1×10^6 PFU / animal intravenously, intraperitoneally and subcutaneously. It was found that 1×10^4 PFU are still able to induce generalized herpesvirus infection in ju-

TABLE 5 Distribution of viral progeny in different organs of tree shrews with generalized herpesvirus infection after injection with recombinant virus HSV-R-HFehx-C19.

Organs	Titer of virus (PFU/g* tissue) Animal	
	juvenile	adult
Blood	7.56×10^3 5.48×10^4	6.25×10^3 5.63×10^4
Brain	1.20×10^1 9.80×10^1	1.43×10^4 4.60×10^4
Liver	5.78×10^4 2.86×10^5	7.00×10^5 1.50×10^6
Spleen	2.24×10^2 1.18×10^4	2.72×10^4 1.29×10^5
Kidney	<1 <1	4.48×10^3 2.98×10^4
Pancreas	<1 <1	2.00×10^2 3.92×10^3
Adrenal glands	3.47×10^5 8.68×10^7	2.88×10^5 1.44×10^7
Testis	ND ND	1.53×10^3 2.55×10^4
Ascites	6.50×10^3 3.33×10^4	ND ND

*PLAQUE FORMING UNITS/GRAM TISSUE; THE RESULTING TITERS OF VIRUS OBTAINED FROM THE PLAQUE ASSAY WERE NORMALISED TO 1.0 GRAM OF THE CORRESPONDING ORIGINAL TISSUE.
ND = NOT DONE

venile tree shrews. In contrast those animals which were inoculated with 1×10^3 PFU or less survived the infection. The possibility that the latent infection can occur in surviving animals was investigated by cocultivating spinal cord, brain, spleen, and other organs. Surprisingly, infectious virus was recovered only from spinal cord of these animals, indicating that the target organ for viral latency for these animals is the ganglia of central nervous system in contrast to its parental virus strain (HSV-1 strain HFEM), which can only be recovered as a latent virus from the spleen of the tree shrews. Genome analysis of the isolated viruses from the spinal cord

of the animal was carried out using restriction enzyme analysis. No changes in the DNA fragmentation patterns of these isolates were detected.

Localization of the deletion

For mapping the deleted genome region of HSV-1 strain HFEM which was apathogenic in mice and tree shrews the Bam HI DNA fragment B of HSV-1 strain HFEM, HSV-1 strain F, and the recombinant HSV (HSV-R-HFehx-C19, C26, and C15) were analysed in detail. A variety of recombinant plasmids, as listed in Table 6, was established which harbour the Bam HI DNA fragment B of HSV-1 strain HFEM (pHSHF-B-B), HSV-1 strain F (pHSF-B-B), and of the recombinant virus HSV-R-HFehx-C19 (pHSR19-B-B), HSV-R-HFehx-C26 (pHSR26-B-B), and finally HSV-R-HFehx-C15 (pHSR15-B-B). The DNAs of the recombinant plasmids were digested with the restriction endonuclease Bam HI and the resulting cleavage patterns were compared to the corresponding Bam HI cleavage patterns of individual donor HSV genomes. It was found that the insert of each recombinant plasmid migrated at the position of the Bam HI DNA fragment B of the parental viral DNA. The identity of the inserts of the recombinant plasmids to its original viral genome and its DNA sequence homology to each other was confirmed using Southern blot hybridization of ^{32}P-labelled pHSHF-B-B DNA to Bam HI digested DNAs of HSV-1 strain HFEM, HSV-1 strain F, and HSV-R-HFehx-C19 as well as to the DNAs of pHSF-B-B and pHSR19-B-B. The results of this analysis are shown in Figure 1A. The data revealed that the DNA of the recombinant plasmid pHSHF-B-B harbouring Bam HI DNA fragment B of HSV-1 strain HFEM hybridized to the Bam HI DNA fragment B of HSV-1 strain F (lane 3), HSV-1 strain HFEM (lane 5), recombinant virus HSV-R-HFehx-C19 (lane 8), and the Bam HI DNA fragment B of HSV-1 strain 17 (lane 1), which served as internal marker. Positive hybridization was found to the DNA sequences of the Bam HI insert of the recombinant plasmids pHSF-B-B (lane 4), pHSR19-B-B (lane 7), and to HSHF-B-B (lane 6). As shown in Figure 1A the ^{32}P-labelled DNA of the plasmid pHSHF-B-B hybridized to the Bam HI DNA fragment E of HSV-1 strain 17 (lane 1), HSV-1 strain F (lane 3), HSV-1 strain HFEM (lane 5)

Table 6 Properties of individual recombinant plasmids.

Recombinant plasmid	Corresponding No. to panel C, Fig.7	DNA fragment of HSV-1(strain)	Insert: inserted in/ vector	Insert: Size of insert (Md/kbp)	Insert: Viral map unit
pHSF-B-B	1	B (F)	in Bam HI of pAT153	6.95/10.5	0.738-0.809
pHSHF-B-B	1	B (HFEM)		4.23/ 6.4	0.738-0.763 & 0.789-0.809
pHSR19-B-B	1	B (R19)*		6.68/10.1	§)
pHSR26-B-B	1	B (R26)*		6.75/10.2	§)
pHSR15-B-B	1	B (R15)**		6.75/10.2	§)
pHS17-B-B	1	B (17)+		6.95/10.5	0.738-0.809
pHSAK-B-B	1	B (AK)+		6.78/10.25	§)
pHSBJ-B-B	1	B (F-BJAB)+		6.78/10.25	§)
pHS1618-B-B	1	B (T-1618)++		6.88/10.4	§)
pHSF-B-Bdel/K	2	B (F) Kpn I deleted		5.23/ 7.9	0.738-0.786 & 0.802-0.809
pHSF-B-Bdel/H(P)	3	B (F) Hpa I(P)deleted		4.37/ 6.6	0.738-0.762 & 0.786-0.809
pHSF-B-Bdel/H(PW)	4	B (F) Hpa I(PW)deleted		3.58/ 5.4	0.738-0.754 & 0.786-0.809
pHSF-B-Bdel/M	5	B (F) Mlu I deleted		2.91/ 4.4	0.738-0.755 & 0.796-0.809
pHSHF-B-Bdel/M	5	B (HFEM) Mlu I deleted		2.91/ 4.4	0.738-0.755 & 0.796-0.809
pHSR19-B-Bdel/M	5	B (R19) Mlu I deleted		2.91/ 4.4	0.738-0.755 & 0.796-0.809
pHSF-B-Bdel/S	6	B (F) Sal I deleted		0.79/ 1.2	0.738-0.746
pHSF-M-C1	7	(F)	in Mlu I of pλ-CE-Mlu I$	3.45/5.22	0.761-0.796
pHSHF-M-C4	8	(HFEM)		0.74/1.12	0.761-0.763 & 0.789-0.796
pHSR19-M-C1	9	(R19)		3.19/4.82	§)
pHSR15-M-C6	10	(R15)		3.39/5.12	§)

* derived from pathogenic recombinant viruses HSV-R-HFehx-C19 and C26.
** derived from apathogenic recombinant virus HSV-R-HFehx-C15.
\+ derived from apathogenic HSV-1 strains AK and F-BJAB which were kindly provided by Professor Kleinschmidt, Department of Virology, University of Ulm, FRG.
\++ derived from apathogenic HSV-T-1618 (Darai et al. 1982)
§ exact position of the deletion not yet determined.
$ pλ-CE-Mlu I vector which contains one Mlu I recognition site was constructed by insertion of phage lambda DNA (λc I ind ts 857 Sam 7; bp: 16126-17791 & 20853-21225 in Eco RI / Cla I sites of pAT153).

and of the recombinant virus HSV-R-HFehx-C19 (lane 8). This result clearly demonstrates the identity and the origin of the inserted Bam HI DNA fragment B of the individual recombinant plasmids to the corresponding region of the HSV-1 genome.

In order to determine the position of the deletion in the viral genome a variety of restriction endonucleases was used. The results of these experiments are shown in Figure 5.
The recombinant plasmids pHSF-B-B, pHSHF-B-B, pHSR19-B-B, pHSR26-B-B, and pHSR15-B-B were cleaved with restriction enzyme Hpa I and the resulting DNA fragments were separated by agarose slab gel electrophoresis. Three DNA fragments were generated after cleavage of pHSF-B-B with Hpa I. In contrast, when the pHSHF-B-B plasmid was analysed under the same conditions, only two DNA fragments were generated. The absence of the Hpa I DNA fragment P revealed that the deletion in the Bam HI DNA fragment B of HSV-1 strain HFEM maps in the Hpa I DNA fragment P of the corresponding intact viral genome. The generation of the Hpa I DNA fragment W (0.754 to 0.762 viral map units) in the case of all recombinant plasmids after digestion with Hpa I indicates that the recognition site of Hpa I at the 0.762 map units of the viral genome is intact. The restriction endonuclease Sal I recognizes two positions in the DNA of the insert of all five recombinant plasmids corresponding to the 0.746 and 0.790 map units of the intact viral genome. This indicates that the Sal I recognition site at the 0.790 map units is present in all DNA species. Further analysis revealed that the deleted DNA sequences in the genome of HSV-1 strain HFEM are spanning map coordinates 0.762 to 0.790 of the HSV-1 DNA (Rösen & Darai 1985). The size of the deletion was found to be 4.1 kbp corresponding to 2.7 Md. The DNA of the pHSR19-B-B plasmid was analysed under the same conditions. It was found that the DNA of this plasmid has three recognition sites for Hpa I enzyme similar to the pHSF-B-B plasmid, but different from pHSHF-B-B which has only two cleavage sites for this enzyme. The presence of the Hpa I DNA fragment P of the pHSR19-B-B plasmid with the size of 3.45 kbp indicates that the deleted region in the genome of HSV-1 strain HFEM was substituted by about 3.45 kbp of corresponding DNA sequences of the Bam HI DNA fragment B of HSV-1 strain F.

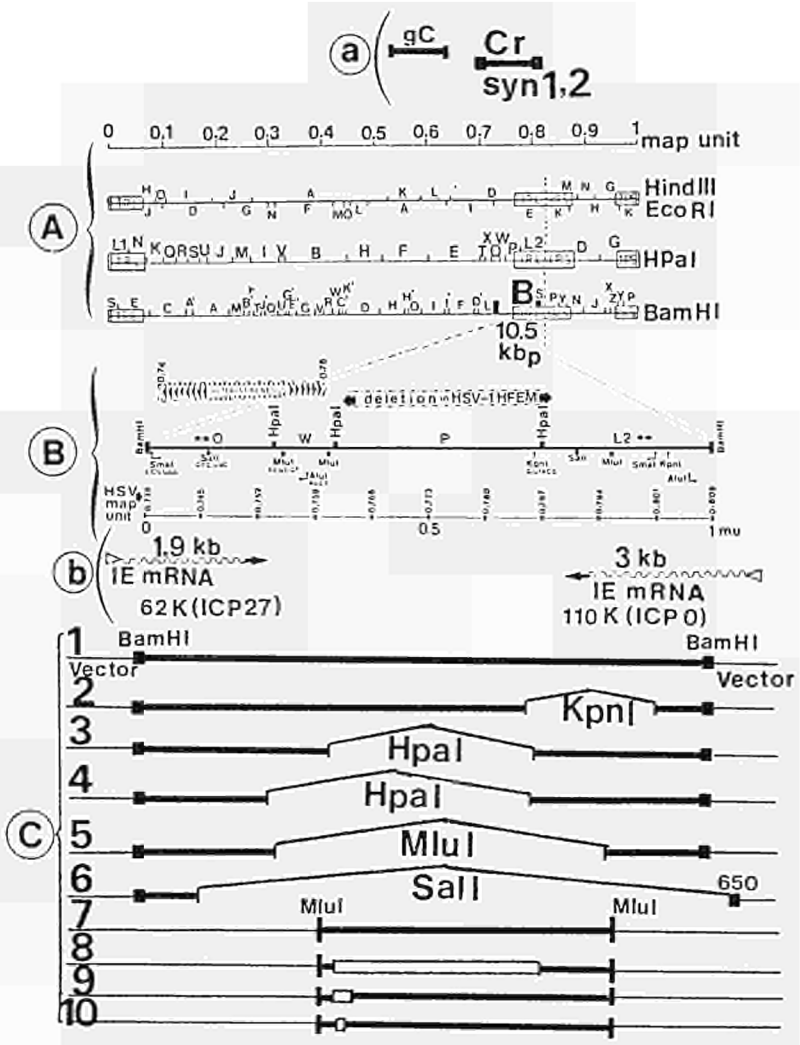

Fig.5 Identification of the deletion in the genome of HSV-1 strain HFEM and its map location. DNA sequence arrangements across the Bam HI DNA fragment B of HSV-1 strain F, determined using recombinant plasmid pHSF-B-B. Panel A: Map of the genome of HSV-1 strain F for Bam HI (Mocarski & Roizman 1981) and for Hind III, Eco RI, and Hpa I (Morse et al. 1977). Panel B: The location of the deletion in the genome of HSV-1 strain HFEM based on the results presented here, and the location of heterogenicity based on the results previously published by Locker & Frenkel

(1979). The position and direction of the arrows for restriction endonuclease Sma I show the external limitation of recognition sites. Between the two marked positions eight additional fragments exist, whose positions are not yet determined. The position and direction of the arrows for restriction endonuclease Alu I show the internal limitation of recognition sites. Beside these positions there is a variety of recognition sites for this enzyme. Panel C: Constructed recombinant plasmids (for detail see Table 6). The localization of the gC and syn phenotypes (panel A) is based on results described by Pogue-Geile et al. (1984) and the position of IEmRNAs and ICP 0 and 27 (panel B) are based on results described by Jacquemont et al. (1984).

DISCUSSION

The experimental approach and strategy for the determination of the genome region responsible for virulence of HSV-1 in vivo used in this report and the results obtained demonstrate that the genetic information located on the DNA sequence region of Bam HI DNA fragment B at the coordinates between 0.738 and 0.809 map units probably seem to be involved in the biological process which has influence on the HSV-1 virulence. The hypothesis based on the facts that the replacement of the deleted Bam HI DNA fragment B of the apathogenic HSV-1 strain HFEM with the corresponding cloned Bam HI DNA fragment B of the pathogenic HSV-1 strain F led to the selection of pathogenic intratypic recombinants. The pathogenicity of the recombinant viruses HSV-R-HFehx-C19 and C26 is reflected in an induction of lethal generalized herpesvirus infection in tree shrew. An infectious dose of 1×10^4 PFU of recombinant C19 is still able to induce fatal viral infection in juvenile tree shrews. In contrast, an infectious dose higher than 1×10^7 PFU of HSV-1 strain HFEM, its subclones and/or clones derived after DNA transfection with native viral genome are still apathogenic in tree shrew.

Furthermore it was observed that the ganglia of spinal cord is the target organ for persisting of latent recombinant virus HSV-R-HFehx-C19, a property known for wildtype HSV-1 and 2, but in contrast to the apathogenic HSV-1 strain HFEM, which can only be recovered as a latent virus from the spleen of the tree shrews. It is known that two of the five major alpha-genes, namely ICP 0 and ICP 27 and two immediate early mRNAs with a

molecular weight of 1.9 and 3 kb (Jacquemont et al. 1984) as well as the Cr and syn 1 and 2 loci are located at this region (Pogue-Geile et al. 1984).
One other interesting aspect is that the deletion in the genome of HSV-1 strain HFEM is not located at the region which is known for occurrence of variabilities. A heterogenicity within U_L DNA sequences spanning map coordinates 0.74 to 0.76 of the HSV-1 genome has been reported (Locker & Frenkel 1979).
Although the replacement of the deletion in the genome of the apathogenic HSV-1 strain HFEM using cloned viral DNA of the corresponding genome region of the pathogenic HSV-1 strain F led to isolation of recombinant viruses with an acquired virulent phenotype, the molecular mechanisms and the functional activities of the viral genes at this genome region in the process of pathogenicity of HSV-1 in vivo are still unclear. Therefore further molecularbiological and biological studies, e.g. the genomic characterization of the Bam HI DNA fragment B of pathogenic and apathogenic recombinants and their parental viruses using heteroduplex mapping, the determination of the DNA sequences, the analysis of their transcripts and gene products are necessary for understanding the molecular mechanisms of tropism and virulence of HSV.

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SUMMARY OF SESSION FOUR.

HERPESVIRUS PATHOGENICITY AND THE IMMUNE SYSTEM.

by G. WITTMANN, R.M. GASKELL and J.T. VAN OIRSCHOT.

In this session dealing with herpesvirus pathogenicity and the immune response very different aspects were handled. REID investigated the pathogenesis and immunity of malignant catarrhal fever. The two distinct agents, alcelaphine herpesvirus 1 and the as yet unidentified "sheep-associated" agent have at least three antigens in common. These agents produce no disease in their natural hosts, wildebeest and sheep, but in other ruminants malignant catarrhal fever almost invariably runs a fatal course. Apart from ruminants the disease can be reproduced experimentally in rabbits, rats and hamsters. In all animals the large granular lymphocyte may represent an important target cell for the virus. It is hypothesized that a virus induced dysfunction of the large granular lymphocytes is primarily responsible for the specific lesions in malignant catarrhal fever, that is characterized by lymphoid accumulations in virtually every tissue, which are distant from areas of necrosis.

CAUCHY, MAZZELLA, COUDERT and RICHARD gave a report on the characterization of T cells transformed by infection with Marek's disease (MD) virus by using monoclonal antibodies. The preparation of monoclonal antibodies against thymus cell membranes of chicken was described. Three clones have been found to react only with cells of the cortical and medullary areas of the thymus and two clones were reactive with certain subpopulations of thymocyte blood T-cells originating from MD tumors. Thus it was demonstrated that MD cell lines and MD tumor cells have a membrane determinant which is present only on mature chicken T-cells.

Another paper presented by WITTMANN and OHLINGER surveyed the present knowledge on humoral and cell-mediated immunity (CMI) to Aujeszky's disease virus (ADV) in pigs. The following parameters were examined: neutralizing antibodies (Ab), spontaneous cell-mediated cytotoxicity against non-infected (SCC) and ADV-infected target cells (ADV-SCC), antibody-dependent ADV-specific cell-mediated cytoxicity (ADV-ADCC), ADV-specific lymphocyte stimulation (ADV-LYST) and interferon production. The state of immunity before and after ADV infection is described with unvaccinated and vaccinated pigs without and with colostral Ab, and with latently infected pigs. It is concluded that ADV-SCC may be of importance for the initiation of infection. Nothing has been known with regards to T-cell participation during this phase. Direct Ab/virus interactions, complement-dependent cytotoxic effects of Ab and ADV-ADCC apparently may have some influence in the latter phase of infection and in recovery. In vaccinated pigs and in pigs with colostral Ab, Ab evoked reactions are primarily involved in protection; ADV-SCC may also play a role.

WELLEMANS, VAN OPDENBOSCH and ANTOINE reported on BHV-4 isolation from cattle with post parturient metritis. The virus could be isolated several weeks after the onset of symptoms. The animals did not have neutralizing antibodies (Ab), but Ab could be demonstrated by the indirect immunofluorescence test and by ELISA and a distinct booster effect was found after parturition. The phase of replication of the virus is unknown and it is concluded that disturbed immunological functions exist in the animals.

Studies of virulence genes of herpes simplex virus (HSV-1) were presented by DARAI and ROSEN. The location of the gene(s) responsible for the virulence of HSV-1 in tree shrews is known. Marker rescue experiments were performed by the co-transfection technique using native DNA of an avirulent HSV-1 strain and the Bam HI DNA fragment R which was derived from a pathogenic strain of HSV-1 and cloned in a bacterial vector. Two out of nine recombinants proved to be lethal for tree shrews. The recombinants proved to be stable. For use in vaccination it seems possible to produce genetechnologically virus which lacks the genome region coding for virulence but contains all the genes responsible for immunogenicity.

SESSION 5

HERPESVIRUS IMMUNITY AND REACTIVATION

Chairman : T.J. HILL

Co-chairman : P.-P. PASTORET

MECHANISMS OF HERPES VIRUS LATENCY AND REACTIVATION WITH PARTICULAR REFERENCE TO THE IMMUNE RESPONSE

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ABSTRACT

The mechanisms underlying establishment, maintenance and reactivation of latent infections with herpesviruses are considered, with herpes simplex virus (HSV) as the primary example. Particular attention is given to the role of the immune response at these different stages of latency. It is concluded that immune mechanisms may prevent access of the virus to the 'target' cell in which latency can be established. However once established it seems unlikely that immune responses are important in maintaining the virus/cell interaction during latency. Following reactivation when infectious virus is produced immune mechanisms are again likely to be involved in clearance of virus from the tissues and thereby in determining whether or not recurrent disease is manifest.

INTRODUCTION

A number of recent reviews have dealt extensively with herpes latency, particularly with respect of EBV (Henle and Henle, 1982) and herpes simplex virus, HSV (Hill, 1984). Therefore in accord with the theme of this symposium, the present paper will focus particularly on the involvement of immunological mechanisms in the different stages of latent infection. Herpes simplex virus (HSV) will be discussed as the prime example but many of the general principles are applicable to other herpesviruses.

Herpesviruses establish a latent infection in particular "target" cells - with alphaherpesviruses such as HSV the main "target" appears to be the sensory neuron but with gamma herpesvirus (CMV), the "target" cell is the lymphocyte. Evidence for latent infection in neurons is strongest for HSV (reviewed by Hill, 1984). However there are indications that extraneural latency may also occur with HSV in the footpad of the mouse or guinea pig (Al-Saadi et al., 1983; Scriba, 1977) and in mouse skin (Hill et al., 1980). Moreover in another neurotropic alphaherpesvirus, pseudorabies, viral DNA has been detected in lymphoid as well as neural tissue (Gutekunst, 1979). It is important to determine whether such virus in extraneural tissues represents true latency or merely microfoci of productive infection fed from primary latency in the nervous system.

The events that encompass the virus/"target" cell interactions in latency can be divided into three main phases: establishment of latency, maintenance and

reactivation (with or without recurrent clinical disease). At each of these phases the virus/"target" cell interaction might be affected by various "external" factors such as hormones or immune effectors. In any infection where virus persists for long periods (with herpes viruses this is usually for a lifetime), there might be expected to be varied degrees of continued or intermittent virus antigen expression. If such expression does occur immunological mechanisms might indeed be amongst the "external" factors which influence the latent state.

However in the face of the selection pressures of the immune system it is also likely that the virus will have evolved means of avoiding complete elimination by the host's defences. Knowledge of such mechanisms is clearly of importance in understanding the pathogenesis of herpetic disease and in designing rational approaches for immunological intervention in prophylaxis or therapy.

ESTABLISHMENT OF LATENCY

Under natural conditions when a herpesvirus first enters the host it will rarely interact directly with the target cell in which latency is to be established. For example with HSV it is probable that such interaction will follow a period of replication in cells of the skin or mucous membrane. Moreover in natural infections the infecting dose may be very small and therefore initial replication in the peripheral tissues will increase the chance of HSV entering the nerve endings. Therefore increasing the extent of this peripheral virus growth e.g. by immunosuppressive treatments, will tend to increase the incidence of subsequent latent infection (Blyth et al., 1980). In contrast diminishing this growth by, for example, anti-viral treatment or vaccination, will have the opposite effect (e.g., Walz et al., 1976). However since the epithelium of mucous membranes and the skin contain many fine nerve endings HSV may have relatively rapid access to the cytoplasm of the sensory neuron. Hence in the non immune host the rapid, non-specific, defence mechanisms are likely to be of great importance in limiting the extent of virus entry into the neuron. Once inside the nerve ending, i.e. in the cytoplasm of the neuron, HSV will be inaccessble to immune effectors and will remain so during intra-axonal transport to the neuronal cell body in the ganglion.

Little is known of the crucial interactions that must occur between HSV and neuron in the ganglion for latency to be established. Present evidence suggests that these interactions can occur quickly (within 1-2 days), that particular viral genes are important (Watson et al., 1980) and that productive infection is unnecessary. Indeed if productive neuronal infection does occur this is likely to result in cell death. Therefore treatments which enhance such infection have been shown to diminish the

incidence of latency infection and vice versa (Price and Schmitz, 1979). Hence in the neuron itself latency may be established with limited expression of the viral genome. Under such circumstances the immune system would have little or no viral antigens to recognise. Therefore it seems unlikely that immune effectors are involved in "forcing" the virus into a latent state. A more likely role for the immune system at this stage is clearance of virus and infected cells from the inoculation site, the p.n.s. and c.n.s. In the later phases of the primary infection (and perhaps in recurrent disease) an unwanted consequence of the immune response may be tissue damage. In the peripheral tissue, with the exception of stromal disease in the cornea, such damage may be of little significance since tissues such as skin are readily repaired. However immunopathological reactions following HSV infection in the c.n.s. may lead to demyelination (reviewed by Hill, 1983) and repair of such damage is much more limited.

MANTENANCE OF LATENCY

At present the weight of evidence for HSV favours the so-called static state of latency with limited expression of the viral genome (Roizman, 1965). Only one report, as yet unconfirmed, describes a viral antigen (the immediate early polypeptide ICP4) in the nuclei in latently infected neuron (Green et al., 1981). If viral antigens are truly absent particularly from the plasma membrane of latently infected neuron, there would be nothing for immune effectors to recognise. Hence such effectors are unlikely to be involved in maintenance of latency. A role for anti-HSV immunoglobulin has been suggested in maintaining latency (Stevens and Cook. 1974). However this seems unlikely since in mice latent infection with HSV can be maintained in the absence of serum neutralising antibodies (Sekizawa, 1980). These animals showed seroconversion when the infection was reactivated. Factors which may be involved in the maintenance (and establishment) of latency are expression of particular viral genes and the physiological state of the "target" cell (highly differentiated and non dividing in the case of neurons). Moreover it has been suggested that the methylation of the viral and/or host DNA may also be involved in maintaining the latent state (Hill, 1984). Preliminary evidence supports this concept since the rate and incidence of isolation of HSV from latently infected ganglia can be increased in vitro by treatment with 5 azacytidine or DMSO (Whitby, Blyth and Hill, unpublished results). Both these drugs cause hypomethylation of DNA.

In the case of lymphotropic herpesviruses such as EBV, there is clear evidence that latently infected cells can express surface viral antigens (e.g. LYDMA) and thereby the cells are made susceptible to attack by cytotoxic T cells (Henle and

Henle, 1982). However lymphocytes consist of a dynamic population of cells which is renewed from the bone marrow. Therefore there may be compensation for the latently infected cells that are lost through the action of immunological effectors. Mechanisms for the maintenance of the number of lymphocytes latently infected with EB virus have been proposed (Rickinson, 1983). Unlike lymphocytes, sensory neurons cannot be replaced. Therefore to avoid loss of latently infected neurons by immune surveillance, the genome expression of neurotropic viruses such as HSV may be more limited in neurons than EB virus expression in latently infected lymphocytes.

REACTIVATION OF LATENT INFECTION

Reactivation is the process by which infectious virus emerges from latent state. A wide variety of stimuli have been shown to induce such reactivation in latently infected experimental animals (Hill, 1984). Some of these stimul e.g. nerve section are known to induce metabolic changes in the associated neuronal cell body and other reactivating stimuli e.g. trauma to the skin (Hill et al., 1978) may have similar effects. Nerve section itself is known to cause increased transcription of neuronal DNA (Watson, 1974) In these circumstances a close association between neuronal DNA and HSV DNA might result in reactivation of the viral genome.

As mentioned above increased expression of the viral genome which is necessary for reactivation may involve changes in methylation of the DNA (Hill, 1984).

In mice latently infected with HSV very severe immunosuppressive treatments are required to induce reactivation in sensory ganglia. At such high doseages drugs such as cyclophosphamide may cause reactivation by acting directly on the latently infected neuron. However with some alphaherpesviruses e.g. bovine infectious bronchitis virus, moderate doses of immunosuppressive steroids can induce shedding of virus in respiratory secretions (reviewed by Ludwig, 1983). Such shedding may arise from reactivated infection in the nervous system and thereby may indicate a direct role for immune mechanisms in the reactivation process. Alternatively viruses such as IBR may show relatively frequent spontaneous reactivation of latency so that treatment with steroids allows such such reactivation to be manifest as virus shedding in peripheral secretions.

In latently infected mice, treatment with cyclophosphamide prolonged the presence of infectious virus in latently infected superior cervical ganglia after induction of reactivation by nerve section (Price and Schmitz, 1979). Therefore although there is no clear evidence for direct involvement of immune effectors in

the reactivation process itself, once reactivation has occurred immune mechanisms are likely to play a crucial role in clearance of virus from the site of reactivation and the peripheral tissues.

RECURRENT DISEASE

In the case of HSV, reactivation of the latent infection may be followed by shedding of virus in the associated peripheral tissue with or without clinical disease. Experiments with HSV in the mouse ear model (reviewed by Hill, 1984) have confirmed the major sequence of events which lead up to recurrent herpes simplex: a variety of peripheral stimuli (all producing inflammatory responses in the skin) induce reactivation in the ganglion, one or two days later virus appears in the skin and in some cases clinical lesions develop (Harbour et al., 1983). Nerve section experiments have shown that virus travels from the ganglion to the skin via the nerve (probably by orthograde axonal flow) (Hill et al., 1983).

Are immunological defects important in allowing recurrent disease to develop? A broad spectrum of such defects are produced by immunosuppressive treatments and in man immunosuppression clearly produces increased severity and probably increased incidence of herpes simplex lesions (Ho, 1977). In the mouse it has proved difficult to induce recurrent disease by short term general immunosuppression alone but, as in man, recurrent lesions which occur during such treatment take longer to heal (Blyth et al., 1980).

Apart from these general effects of immunosuppressive treatments, "naturally" occurring minor defects in T cell surveillance may play a part in recurrent herpetic disease (reviewed in Rouse and Lopez, 1984). In particular reduced number of MIF producing lymphocytes have been found in the circulation at the time of recurrent disease. These changes are paralleled by an increase in the number of suppressor T cells. However it is difficult to be sure whether such changes are the cause or the effect of recurrent herpes simplex. Moreover caution is required when interpreting studies of human peripheral blood since the crucial changes in immunoregulation may be detectable only in the locality of the infected peripheral tissue and/or the draining lymph nodes. The importance of these more local changes have been stressed in the "skin trigger" theory of recurrent disease (Hill and Blyth, 1976; Hill, 1984). In this theory it is envisaged that the stimuli (such as U.V. light, or trauma) which induce recurrent herpes simplex, may themselves alter conditions in the skin so as to favour the development of a lesion. Such changes could occur either by direct effects on the susceptibility of skin cells to infection or by transient local immunosuppression. In either case it has been suggested that the release of

prostaglandins may be involved (Hill, 1984; Blyth and Hill, 1984).

IMMUNOLOGICAL INTERVENTION

From this brief review it seems that attempts to control latent herpes infection by immunological intervention may only be possible at two stages of the infection. Firstly at a very early stage so as to prevent interaction of the virus with the "target" cell (neuron in the case of HSV) i.e. to prevent establishment of latency. Secondly, after reactivation so as to remove infectious virus and thereby lessen the chance of virus shedding and recurrent disease. The latter is likely to prove more difficult since recurrent disease usually occurs in an already immune host.

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STUDIES ON CONDITIONS NECESSARY FOR *BOVINE HERPESVIRUS 1* REACTIVATION

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ABSTRACT

The conditions for reactivation of *Bovine herpesvirus 1* were studied in different situations. The spontaneous reactivation of the thermosensitive (ts) strain and of a conventionnally attenuated strain, both intranasally administered, was examined in healthy young cattle: no spontaneous reactivation was demonstrated until 8 months after vaccination. Reactivation of ts strain during parturition was investigated and was shown to be a rare event: no nasal re-excretion occurred and only 1 out of 9 cows showed a rise in BHV 1 neutralising antibodies. Reactivation of ts strain by transport is not a constant feature, but nasal re-excretion was detected in 8 out of 19 animals, 1 to 3 days after transport. *Dictyocaulus viviparus* infestation is able to reactivate the ts strain 20 days after vaccination. This result implies that BHV 1 can be reactivated very soon after primary infection.

INTRODUCTION

The epidemiological significance of the persistence of *Bovine herpesvirus 1* (BHV 1) in a latent state is obviously linked with the possibility of viral reactivation followed by re-excretion of infectious particles.

Several latent herpesviruses can be reactivated by injection of glucocorticoids: this procedure is extensively used in experimental work to reactivate BHV 1 in latent bovine carriers (Sheffy and Davies, 1972). It is also reported that therapeutic use of glucocorticoids provokes the reactivation of BHV 1 in treated cattle (Wellemans et al., 1976). Several other stimuli can also provoke reactivation: superinfection with para-influenza 3 virus (Mensik et al., 1976), oral administration of 3-methylindole (Espinasse et al., 1983), *Dictyocaulus viviparus* infestation (Msolla et al., 1983).

It is usually assumed that stressful conditions can lead to BHV 1 reactivation and this accounts, for example, for infectious bovine rhinotracheitis (IBR) outbreaks in recently assembled cattle, in which re-excretion of infectious virus by a latent carrier propagates a virulent strain to uninfected cattle.

Spontaneous reactivation of field virus occurs more frequently in the

genital tract than in the respiratory system. The term "spontaneous" must be cautiously interpreted: in this paper, spontaneous reactivation or re-excretion will refer to reactivation with or without re-excretion induced without the aid of external identifiable stimuli such as those described above.

Spontaneous genital re-excretion of field virus is often demonstrated in both sexes, even more than one year after primary infection (Snowdon, 1965; Bitsch, 1973), but the amount of virus is lower during re-excretion than after primary infection (Bitsch, 1975). Spontaneous nasal re-excretion of wild-type strains is a rarer event (Snowdon, 1965; Bitsch, 1973), but, in dairy herds, seroconversion against BHV 1 and nasal isolation of field virus have been regularly demonstrated, indicating the circulation of virus in the herd, following its reactivation in one or more animal (Hyland et al., 1975; van Nieuwstadt and Verhoeff, 1983).

Other circumstances that are theoretically able to induce BHV 1 reactivation have received little or no attention. In addition, the conditions of reactivation of vaccine virus in the field have not yet been extensively investigated. We therefore examined the spontaneous reactivation of the thermosensitive (ts) vaccine strain and of a conventionnally attenuated strain in healthy young cattle. Thereafter, parturition, transport and *Dictyocaulus viviparus* infestation were evaluated as possible causes of reactivation of the ts vaccine strain in the field.

SPONTANEOUS REACTIVATION OF TWO BHV 1 VACCINE STRAINS ADMINISTERED INTRANASALLY (Thiry et al., 1983)

In dairy herds, reactivation of virulent BHV 1 regularly occurs and provokes episodes of nasal re-excretion and seroconversion (Hyland et al., 1975; van Nieuwstadt and Verhoeff, 1983). The possibility of spontaneous reactivation of vaccine strains administered intranasally was studied in a selection station where animals are kept under optimal conditions.

Twenty-eight 3-months-old bullcalves were followed until their slaughter, at the age of 1 year. None of them excreted BHV 1 at day 0 (day of vaccination); three calves had low titres of BHV 1 neutralising antibodies (2 or <2). They were divided into four groups and were vaccinated with two live vaccines, Tracherine (ts vaccine strain, Smith Kline, R.I.T.) and IBR-IPV (Bayer), following the procedures recommended by the manufacturers.

	bulls n°	day 0	day 27
Group 1	1 to 7	Tracherine	IBR-IPV
Group 2	8 to 14	Tracherine	Tracherine
Group 3	15 to 21	IBR-IPV	Tracherine
Group 4	22 to 28	IBR-IPV	IBR-IPV

After the first vaccination, 13 out of 14 ts strain-vaccinated calves excreted the vaccine virus and 10 out of 14 IBR-IPV strain-vaccinated animals excreted BHV 1. The excretion of ts strain was demonstrated by virus titration at 35°C and restriction of virus multiplication at 40°C. After the second vaccination, no bulls from groups 1 and 3 excreted the virus; in group 2, 3 out of 7 animals and in group 4, 4 out of 7 animals excreted the virus. Nasal swabs were taken monthly during eight months: no virus excretion was detected in any case (table 1).

TABLE 1.- Nasal excretion and re-excretion of vaccine viruses (number of animals which (re)excrete BHV 1)

days: / groups:	0 to 7 first vaccin.	27 to 34 second vaccin.	77	112	167	216	244
1	13/14	0/7	0	0	0	0	0
2		3/7	0	0	0	0	0
3	10/14	0/7	0	0	0	0	0
4		4/7	0	0	0	0	0

Seroneutralisation tests performed on samples taken after vaccination revealed that 10 out of 28 animals did not show any seroconversion; seroneutralisation was performed by a micromethod previously described (Thiry et al., 1983).

TABLE 2.- Prevalence of calves possessing BHV 1 neutralising antibodies (titre ≥ 2)

days: / groups:	0	27	77	112	167	216	244
1	0/7	3/7	7/7	0	0	0	0
2	2/7	1/7	4/7	0	0	0	0
3	0/7	1/7	3/7	1/7	0	0	0
4	1/7	1/7	4/7	0	0	0	0

Only one animal was still seropositive at day 112 and, afterwards, all the animals were seronegative (table 2).

No spontaneous reactivation of vaccine virus was demonstrated in these animals until 8 months after vaccination: neither nasal re-excretion nor a rise in BHV 1 neutralising antibodies were detected. This situation is in sharp contrast to what is observed in dairy herds, as indicated above. In our study, animals were not submitted to stressful conditions such as bad management or introduction of new animals; they had all the same age and did not develop any infectious pathology during the observation period. Under these conditions, spontaneous reactivation of BHV 1 vaccine virus administered intranasally is at least a rare event.

REACTIVATION OF INTRANASALLY ADMINISTERED TS VACCINE STRAIN DURING PARTURITION (Thiry et al., 1984)

Blood levels of glucocorticoids increase before and during parturition in the bovine species (Smith et al., 1973). The intensity of labour also influences the level of corticoids (Hudson et al., 1976). Parturition could thus represent a circumstance able to reactivate BHV 1 in a pregnant cow, latent carrier of the virus. This possibility was investigated in 9 pregnant cows from a herd of 43 Holstein Friesian cows belonging to an agricultural Institute. These cattle had never been vaccinated against IBR. No respiratory disease had occurred on the farm during the last years and only a few animals possessed low titres of BHV 1 neutralising antibodies (titres: 35/43: 0; 7/43: 2; 1/43: 4). All the animals were vaccinated with Tracherine (ts vaccine strain) following the recommended dosage. Nasal mucus samples were taken every 2 days, beginning 4 days before the theoretical date of parturition until the sixth day after parturition; sampling could not be continued for practical reasons. Plaque assay was performed in cell culture covered by Minimum Essential Medium (MEM) supplemented with 2.5% anti-BHV 1 bovine immuneserum (neutralising titre: 64). Either a field strain latently carried by cows at the time of vaccination or the vaccine strain could be reactivated in these animals.

No infectious BHV 1 particles were detected in any swab by plaque assay at 35 and 40°C. Seroneutralisation was performed by a micromethod previously described (Thiry et al., 1983). Only one cow (171) presented a significant rise in BHV 1 neutralising antibodies after parturition (table 3), but without re-excretion of infectious particles, although its immune status was low at the moment of parturition, as indicated by seroneutralisation.

TABLE 3.- BHV 1 neutralising antibody titres in cows after parturition

animals:	day of vaccination	day of parturition	one week later	two weeks later
27	2	16	16	16
31	2	2	2	2
34	0	< 2	< 2	< 2
35	2	2	2	2
36	2	< 2	< 2	4
38	0	4	8	8
45	0	< 2	< 2	< 2
47	0	4	4	4
171	0	2	2	8

Even if this seroconversion reveals BHV 1 reactivation without re-excretion of infectious particles, it can therefore be concluded that reactivation of ts strain occurs only rarely during parturition. Parturition itself seems not to be a major cause of BHV 1 reactivation.

REACTIVATION OF INTRANASALLY ADMINISTERED TS VACCINE STRAIN BY TRANSPORT

Transport is generally considered as a possible cause of BHV 1 reactivation. In fact, it is associated with an increase in corticoid blood levels in bovine and used to simulate stressful conditions in this species (Völker et al., 1973; Johnston and Buckland, 1976; Kent and Ewbank, 1983).

The experiment was carried out in five parts, depending on the opportunities of transport of heifers belonging to an embryo transfer research unit. Fourty-six animals of this unit were vaccinated with a double dose of Tracherine, the ts vaccine strain. At the moment of vaccination, the distribution of BHV 1 neutralising antibody titres was: 14/46:0; 25/46: 2; 2/46: 4; 2/46: 8; 1/46: 16; 1/46: 32; 1/46: 128.

Two months after vaccination, two groups of animals (group 1: 5 animals; group 4: 6 animals) were transported over a distance of 170 km. Six months after vaccination, three groups of animals were transported: group 2 (6 animals, 90 km); group 3 (8 animals, 140 km); group 5 (8 animals, 200 km). Nasal swabs were taken the day of transport (just before or just after transport) and then regularly until the sixth or the ninth day. They were stored at -70°C. The presence of infectious particles was examined by plaque assay at 35 and 40°C in cell culture covered by MEM

supplemented with 2.5% anti-BHV 1 bovine antiserum. Serum samples were taken the day of transport, then 1 and 2 or 3 weeks later. BHV 1 neutralising antibodies were titrated by a micromethod (Thiry et al., 1983). In groups 4 and 5, only serum samples were collected. It must be emphasized that either the vaccine strain or a field strain latentlty carried by cattle at the time of vaccination could be reactivated.

The results of this experiment are given in tables 4 to 8. In groups 1 to 3, re-excretion of infectious particles was demonstrated in 8 out of 19 animals from days 1 to 3 after transport; only low titres were observed and reactivated viruses multiplied only at 35°C. No significant rise in neutralising antibodies occurred, although each animal possessed BHV 1 neutralising antibodies (tables 4 to 6). In groups 4 and 5, in which sera were examined, 1 out of 14 animals (n°26) showed a significant rise in BHV 1 neutralising antibodies (tables 7 and 8).

TABLE 4.- Transport of group 1 animals.
(170 km; 2 months after vaccination)

	PFU/ml in nasal swabs at 35°				BHV 1 neutralising antibody titre			
days:	0	2	3	6	day of vaccination	0	6	16
animals:								
1	0	0	0	0	0	< 2	0	2
2	0	0	0	0	0	2	4	4
3	0	0	0	0	16	16	16	32
4	0	40	0	0	0	8	8	16
5	0	100	90	0	0	2	2	2

day 0: day of transport.

TABLE 5.- Transport of group 2 animals.
(90 km; 6 months after vaccination)

	PFU/ml in nasal swabs at 35°						BHV 1 neutralising antibody titre			
days:	0	1	2	4	5	8	day of vaccination	0	8	20
animals:										
6	0	0	0	0	0	0	4	8	8	8
7	0	0	0	0	0	0	2	8	8	16
8	0	0	0	0	0	0	0	2	2	2
9	0	70	0	0	0	0	2	2	2	0
10	0	0	0	0	0	0	2	2	2	4
11	0	100	0	0	0	0	2	2	2	2

TABLE 6.- Transport of group 3 animals.

(140 km; 6 months after vaccination)

	PFU/ml in nasal swabs at 35°						BHV 1 neutralising antibody titre			
days:	0	1	2	3	6	9	day of vaccination	0	6	16
animals:										
12	0	0	0	0	0	0	2	2	2	2
13	0	0	0	30	0	0	4	16	16	8
14	0	0	0	0	0	0	2	2	2	2
15	0	0	0	0	0	0	2	2	2	2
16	0	0	0	0	0	0	2	2	2	2
17	0	100	40	120	0	0	2	8	8	8
18	0	40	20	80	0	0	2	4	2	4
19	0	80	0	0	0	0	0	2	2	0

day 0: day of transport

TABLE 7.- Transport of group 4 animals; BHV 1 neutralising antibody titres.

(170 km; 2 months after vaccination)

days:	day of vaccination	0	4	14	18
animals:					
20	0	0	0	0	0
21	0	4	4	4	2
22	0	4	4	4	2
23	0	8	16	16	8
24	0	< 2	2	2	2
25	0	2	2	2	2

TABLE 8.- Transport of group 5 animals; BHV 1 neutralising antibody titres.

(200 km; 6 months after vaccination)

days:	day of vaccination	0	8	18
animals:				
26	8	16	32	128
27	2	0	2	2
28	0	0	2	2
29	< 2	0	2	2
30	0	2	2	2
31	< 2	2	2	2
32	2	4	4	2
33	0	2	2	0

Reactivation of BHV 1 by transport was not a constant feature, but occurred in 8 out of 19 cows, at least in our conditions of work: cattle vaccinated with ts strain, young adult cattle kept under excellent hygienic conditions (embryo transfer research unit). When re-excretion occurred, a limited amount of infectious particles were excreted, over a short period (1 to 3 days): this is obviously not the best way to transmit the virus to in-contact cattle.

REACTIVATION OF INTRANASALLY ADMINISTERED TS VACCINE STRAIN BY *DICTYOCAULUS VIVIPARUS* INFESTATION

It has been recently reported that virulent BHV 1 can be reactivated in cattle experimentally infested with *Dictyocaulus viviparus* larvae (Msolla et al., 1983): BHV 1 was recovered from days 7 to 21 post-infestation. We have evaluated the possibility of early reactivation in animals vaccinated with ts strain one week before infestation with *D. viviparus*.

Eighteen 3-months-old Friesian calves were vaccinated at day 0 with a double dose of Tracherine (ts vaccine). At day 0, 5 calves possessed BHV 1 neutralising antibodies (table 10). At day 8, they were orally infested with *D. viviparus* L_3 larvae (30 L_3/ kg body weight). At day 15, the animals were divided into 3 groups: the first group was treated once with levamisole (10 mg/kg; subcutaneous injection); the second was treated once with ivermectine (200 µg/kg; subcutaneous injection); the third was an untreated control group. Nasal swabs were taken twice a week during 7 weeks and serum samples once a week. Infectious viral particles were titrated by plaque assay as described above, at 35 and 40°C. Either the ts strain or a field strain present in these calves in a latent stage could be reactivated.

Excretion of vaccine virus was demonstrated in 13 out of 18 vaccinated animals from days 2 to 13 (table 9). The 5 calves in which no excretion was detected possessed BHV 1 neutralising antibodies at day 0. At day 20, 12 days after experimental infestation, re-excretion of BHV 1 occurred in 13 animals at very low titres (table 9). Subsequently (day 44), one animal re-excreted virus at 35°C. Excretion and re-excretion of BHV 1 was demonstrated only at 35°C, indicating in each case that only the ts vaccine virus was present (table 9). Seroconversion was not detected in 3 animals (4, 9, 18). No rise in specific neutralising antibodies was observed after day 20, except in 2 animals (3 and 15) (table 10).

TABLE 9.- Excretion and re-excretion of BHV 1 after vaccination and subsequent *D. viviparus* infestation.

days:		0	2	6	9	13	16	20	23	...	44
animals:											
group 1:	1	0	100	$2x10^3$	0	0	0	170	0		0
	2	0	$4.4x10^3$	10^4	$7x10^3$	$2x10^3$	0	0	0		0
	3	0	300	10^3	0	0	0	10	0		0
	4	0	$8.5x10^4$	$6x10^3$	0	0	0	0	0		0
	5	0	10^5	10^4	100	0	0	10	0		0
	6	0	$2x10^4$	10^4	$1.5x10^3$	0	0	0	0		0
group 2:	7	0	0	0	0	0	0	70	0		0
	8	0	0	0	0	0	0	70	0		0
	9	0	$1.7x10^3$	$4.6x10^3$	$1.5x10^3$	0	0	30	0		0
	10	0	$1.5x10^4$	10^4	900	0	0	300	0		0
	11	0	$4.8x10^4$	$5x10^3$	30	900	0	20	0		0
	12	0	$3.5x10^4$	$5.1x10^3$	$1.3x10^3$	0	0	80	0		0
group 3:	13	0	0	0	0	0	0	0	0		0
	14	0	0	0	0	0	0	0	0		0
	15	0	$4.3x10^3$	$4.6x10^3$	0	0	0	70	0		0
	16	0	0	0	0	0	0	10	0		0
	17	0	$1.4x10^3$	$4.3x10^3$	600	0	0	30	0		30
	18	0	100	0	400	0	0	60	0		0

titres are given in PFU/ml of nasal swabs at 35°C;
day 0: vaccination; day 8: infestation; day 15: treatment;
group 1: treated with levamisole; group 2: treated with ivermectine; group 3: untreated.

Whatever the treatment applied, re-excretion of ts vaccine strain was demonstrated in each group. We are not in a position to speculate on the differences between groups as far as the number of calves re-excreting virus is concerned: animals not shown to be re-excreting could have re-excreted the virus out of the sampling days. In the same way, the fact that re-excretion was shown in 3 calves that did not excrete after vaccination is not surprising: they could have in fact excreted the vaccine virus out of the sampling days, over a very limited period.

Treatment with ivermectine and levamisole sharply reduced the amount of larvae present in the faeces and the amount of worms in the lungs, as

compared to the control group, but they had not impede the migration of certain larvae through the alveolar wall; it is thus probable that these treatments did not prevent the injury of the respiratory epithelium caused by the passage of larvae. This could represent the stimulus inducing reactivation, because re-excretion of BHV 1 was detected at day 20, 12 days after infestation: this is precisely the moment when larvae penetrate the alveolar walls and enter the lumen.

TABLE 10.- BHV 1 neutralising antibody titres after vaccination and subsequent infestation with *D. viviparus*.

days:		0	9	16	23	30	37	(42-48)
animals:								
group 1:	1	0	0	2	2	2	2	2
	2	0	0	8	16	16	8	8
	3	0	0	2	2	4	2	8
	4	0	0	0	0	0	0	0
	5	0	0	4	8	8	4	4
	6	0	0	4	8	8	8	4
group 2:	7	32	16	16	16	16	16	16
	8	4	8	8	16	8	8	8
	9	2	0	0	2	0	0	0
	10	< 2	0	4	8	8	8	16
	11	0	< 2	2	4	8	4	8
	12	0	< 2	< 2	0	2	2	< 2
group 3:	13	8	4	8	8	8	8	16
	14	16	16	16	16	16	16	32
	15	0	0	< 2	0	4	4	4
	16	16	16	16	16	16	16	16
	17	0	2	8	8	8	8	16
	18	0	0	0	0	0	0	0

day 0: vaccination; day 8: infestation; day 15:treatment.

Except in two cases (3 and 15), no seroconversion was detected after day 20. During reactivation, the animals were still in primary immune response and the stimulus was probably not sufficient to increase the neutralising antibody titres. Calves in which neutralising antibodies rose after day 20 did not shown any seroconversion before. During all the period of

examination, no neutralising antibody was detected in 3 animals, although the multiplication of the vaccine strain was proven in these calves (tables 9 and 10): vaccination with ts vaccine does not usually induce high levels of neutralising antibodies; moreover, certain animals do not show any serological response although they are protected against the clinical manifestations of the disease after challenge with virulent virus (Zygraïch et al., 1974; Nettleton and Sharp, 1980).

Re-excretion is demonstrated very soon after the primary infection (day 20). It is reported elsewhere that reactivation can occur 28 days after primary infection with a virulent strain (Espinasse et al., 1983), or 40 days after primary infection with ts vaccine strain (Pastoret et al., 1980). Attempts to reinfect cattle with virulent strain, four weeks after vaccination with ts strain, are successful, although the level of excretion is reduced (Zygraïch et al., 1974). We may therefore assume that BHV 1 can be reactivated soon after primary infection. The level of re-excretion is decreased probably because the greater part of reactivated virus was neutralised and thereby not detectable as infectious particles. Bitsch (1975) observed that the level of virus excretion was usually lower after spontaneous reactivation than after primary infection.

DISCUSSION AND CONCLUSIONS

The results reported here show that certain conditions which could presumably reactivate BHV 1 in the field are effective reactivation stimuli, at least for the ts vaccine strain. When re-excretion occurs, the amount of re-excreted virus is low and the duration of re-excretion is short, so that transmission of the virus may be difficult even if a high percentage of animals re-excrete the virus, as after transport or infestation with *D. viviparus*. On the contrary, parturition seems to be an inefficient reactivation stimulus for the ts vaccine strain.

We postulate thet these stimuli, and others of the same nature, will induce full reactivation if their effects are combined on the same animal. In fact, if the stimulus is stronger, we reach a situation similar to that of experimental treatment with dexamethasone which, when correctly applied, provokes approximately 100% of reactivation and re-excretion with high virus titres (Pastoret et al., 1980). For example, the increase in blood corticosteroid levels in parturient cows is proportional to the duration and intensity of labour (Hudson et al., 1976). In Holstein-Friesian cows examined in this study, calving problems occur rarely and, except parturi-

tion, these animals were not subjected to other objectivable stressful conditions. Transport is often associated in young calves with the so-called "Shipping fever" syndrom, in which parainfluenza-3 virus is partly involved. Infection with this virus can provoke BHV 1 reactivation (Mensik et al., 1976) and the combination of these stimuli could lead to reactivate BHV 1 easier. In this study, 8 out of 19 animals re-excreted virus after transport: they were two-years-old heifers; no new animals were introduced and no respiratory illness was observed after transport.

When speaking about BHV 1 reactivation, distinction should be made between infection by the nasal or the genital route. Genital infection was the natural situation in cattle before the fifties in U.S.A. and the seventies in European countries. Genital latency has thus been a naturally fixed situation since a long time ago (Bläschenausschlag was first described in 1845) and the interactions between virus, host and environment have evolved to make easier genital reactivation followed by re-excretion, allowing the propagation of the infection in herds. On the contrary, respiratory infection by BHV 1 is a phenomenon of recent appearance in the history of the infection and, perhaps, virus-host-environment interactions have not yet reached the same degree of adaptation as in the case with genital infection. It is thus experimentally established that, following infection with a genital isolate, genital re-excretion is much more frequent than nasal re-excretion (Snowdon, 1965; Bitsch, 1975).

This hypothesis and the fact that we used an attenuated strain give some explanations of the reduction of the number of animals which re-excreted in this study.

In conclusion, the ts vaccine strain is not frequently re-excreted after vaccination in field conditions, although it shows the same nasal re-excretion pattern as virulent strains when dexamethasone treatment is applied on latent carriers (Pastoret et al., 1980; Nettleton et al., 1984). If an appropriate stimulus occurs, the ts strain is re-excreted at low titres, during a short time. The propagation of this vaccine strain is thus limited and probably restricted to the days following vaccination.

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BOVINE HERPESVIRUS 1 : REACTIVATION BY 3-METHYLINDOLE.

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ABSTRACT

3-Methylindole (3-MI) is a powerful pulmonary toxic. After an experimental infection with bovine herpesvirus-1 (BHV-1), 4 out of 6 adult bovines received various intra-ruminal doses of 3-MI. No viral excretion was observed in tracheal or nasal mucus. Immunological response was the only evidence of the BHV-1 reactivation. Seric and local antibody levels increased after each dose of 3-MI.

From the current knowledge on cellular effects of 3-MI, some hypotheses to explain BHV-1 reactivation are discussed.

INTRODUCTION

3-Methylindole (3-MI) is a rumenal fermentation product of L-tryptophan. 3-MI is involved in naturally occurring acute pulmonary edema (fog fever) (Breeze et al., 1975) and oral administration has been shown to cause some lesions in cattle (Carlson et al., 1975), sheep, goats (Bray and Carlson, 1979) and ponies (Derksen, 1982).

Many herpesviruses in various species are liable to persist in a latent form in the host after primary infection. Particularly in cattle, it has been demonstrated that latent bovine herpes virus-1 (BHV-1) can be reactivated by corticosteroid administration (Dennet et al., 1973 ; Sheffy and Davies, 1972 ; Pastoret et al., 1978), experimental infection with Parainfluenza 3 virus (Mensick et al., 1976) or following Dictyocaulus viviparus infestation (Msolla et al., 1983b). In other species, reactivation of latent herpesviruses has been demonstrated using various stimuli : application of dry ice on the lips of Herpes Simplex Virus (HSV)-infected mice (Klein, 1982), immunosuppression of HSV human carrier after organ transplantation (Armstrong et al., 1976), electrical excitation of trigeminal ganglia of infected rabbit (Green et al., 1981), cytotoxic agents (Openshaw et al., 1979).

The aim of this study is to investigate the effect of 3-MI, known to be an important pulmonary toxic, on latent BHV-1 infected cattle.

MATERIALS AND METHODS

Animals

Six heifers, 15 ± 6 months old, of various breeds were raised in individual indoor stalls. They were fed with hay

twice a day. On day 0, each animal was inoculated with $10^{6.1}$ tissue culture infectious dose 50 % (TCID 50) of BHV-1 diluted in 5 ml of Eagles's minimum essential medium supplemented with 2 % foetal calf serum. On day 110, four heifers, A, B, C and D were given intrarumenally 3-MI* melted in 1-2 propanediol. A and B received 25 mg/kg body weight and C and D 50 mg/kg. On day 131 A, B and C, D were given sub-toxic 3-MI doses, respectively 75 mg/kg and 100 mg/kg. 1-2 propanediol was only given on days 110 and 131 to two heifers, E and F.

Clinical examination and sampling

Each animal was examined daily in order to record respiratory and heart rates and rectal temperature. On days 1, 2, 4, 7, 10, 14 and then once a week until the 5th week, animals were sampled by transtracheal aspiration. On days 49 and 56, only nasal swabs were collected. Both transtracheal aspiration and nasal swabbing were undertaken on days 1, 2, 3, 4, 6, 8, 10, 14, 20 after each 3-MI administration; heifers were bled on the same days.

Virology

Each swab was immediately pressed into a sterile, disposable syringe. Mucus was harvested in sterile tubes, centrifuged at 5000 rpm for half an hour. After serial dilution, 0.1 ml aliquots of supernatant fluid were used to infect 96-well microplates**, containing secondary or tertiary foetal calf kidney cells. Each dilution was tested in two wells.

Starting with centrifugation, transtracheal aspirates (TTA) were processed in the same manner.

Neutralization test

BHV-1 serum neutralization tests were performed as described by Bitsch (1978). Serial 3 fold dilutions starting from the tenth were titrated in 96 wells plates on MDBK cells using a calculated 100 BHV-1 TCID 50 dose.

ELISA test

ELISA plates*** were coated overnight with BHV-1 antigen prepared in the laboratory. Plates were washed three times with phosphate buffered saline pH 7.2 containing tween at a concentration of 0.05 % (PBS-Tw). Then they were incubated with a PBS-Tw 1 % gelatine solution for one hour in order to avoid attachment of non specific antibodies. Serial four-fold dilutions of sera, starting from the twentieth were laid onto the plates for twenty minutes. Then they were washed again and incubated with peroxidase-labelled rabbit anti-bovine IgG****. After the last washing sequence, a 0.5 % orthophenylenediamine solution in citrate- phosphate buffer (w/v) added with H_2O_2 (1/400 V/V) was distributed. The activity of the enzyme was stopped with H_2SO_4 2M. Plates were read on an ELISA spectrophotometer with a filter of 492 nm.

Local antibody titration

Nasal swab exsudates were titrated for BHV-1 antibodies as previously described by Le Jan and Asso (1981).

RESULTS

Experimental infection

All six infected heifers developed a transient rectal temperature elevation and a moderate nasal and ocular discharge. Ulcerative and fibrinous rhinitis occurred for C, E and F. A, B and D only showed a congestion of the nasal mucous membrane and conjunctiva. By day 21 post infection (pi), all general and local clinical signs had disappeared. A, B, D, E and F excreted BHV-1 through the trachea from day 2 to 7 pi and C to day 10 pi, reached a maximum between the fourteenth and twentieth days pi, varied to day 70 pi and decreased for the six animals (Fig. 2). ELISA results showed the same pattern as neutralizing antibodies (Fig. 3). Local BHV-1 antibodies were irregularly observed from the tenth to the fiftieth days pi.

3-MI administration

Neutralizing and ELISA antibodies of A and B increased after 3-MI administration. ELISA and neutralizing antibodies did not vary for the reference animals (Fig. 4). The level of neutralizing antibodies also increased after the second 3-MI administration for A and B. ELISA for all animals and neutralizing antibodies for C, D, E and F remained stable (Fig. 5). Low levels of local antibodies were irregularly found in the four tested animals after both 3-MI administrations. BHV-1 was never recovered from nasal swabs from both 3-MI tested and reference animals.

DISCUSSION

The present results do not clarify previous data on the effect of 3-MI on BHV-1 latency (Espinasse et al., 1983). Bovines free of neutralizing and ELISA BHV-1 antibodies have shown usual clinical and immunological reaction after an experimental infection (Msolla et al., 1983a). A former experiment (Espinasse et al., 1983) and the present one give conflicting results.

Viral excretion found previously with 150 mg/kg and 200 mg/kg of 3-MI did not occur with lower doses varying from 25 to 100 mg/kg. In contrast, the moderate increase in antibodies became more evident when lower doses of 3-MI were used. The increasing titers of serum neutralizing antibodies with two low doses (25 mg/kg and 75 mg/kg) was confirmed in five neutralization assays and never accompanied by any virus shedding.

Two hypotheses could be suggested to explain the action of 3-MI:

i) 3-MI behaves as a non specific immune stimulant at lower rather than sub-toxic doses as it was demonstrated with an immune modulator (Floc'h et al., 1983)

ii) 3-MI allows BHV-1 release, which acts as a booster injection, either by destruction of BHV-1-containing cells or by direct activation. Failure to detect BHV-1 could be explained in this last hypothesis by the maintenance of the immune functions wich are not depleted as they could be, by

using corticosteroids. This remains consistent with some evidence that dexamethasone exerts a direct effect on latently infected cells to induce herpesvirus reactivation (Pastoret et al., 1982).

Cornelius (1979) has established, and Hanafy and Bogan (1982) have confirmed, that 3-MI is not the direct cytotoxic product. Nine different metabolites of 3-MI degradation were described in the urine of goats administered with 3-MI (Potchoiba et al., 1982). Two of these were identified as main 3-MI metabolites and no direct action of all of them was clearly demonstrated. The active unknown 3-MI derived toxic was recognized as acting on lung phospholipid synthesis (Kirkland and Bray, 1984) and on the mixed function oxidase system (Bray and Carlson, 1979). Individual susceptibility of cows has been found in various attempts at experimental non lethal reproduction of fog fever (unpublished data). However, in the present experiment, neutralizing antibody increase occurred in both cases in the same heifers. Further experiments could be carried out to amplify and improve our understanding of the described mild 3-MI effect. Nevertheless, in the absence of any inactivated reference antigen, to test possible lymphocytic non-specific activation, BHV-1 reactivation cannot be assumed from this experiment. However, BHV-1 excretion in the former experiment appeared to be spontaneous rather than induced reactivation. These results and the lack of accurate data on 3-MI metabolism and its effect or the effect of related substances on immune functions, make it difficult to reliably evaluate the action of 3-MI on BHV-1 latency.

* 3-Methylindole (skatole), Sigma Chemical Company.
** Falcon microtest II 30-40, Becton Dickinson laboware.
*** Limbro E.I.A. microtitration plate, Flow Laboratories.
**** Anti-bovine IgG (Rb) Peroxidase, Miles Biochemicals.

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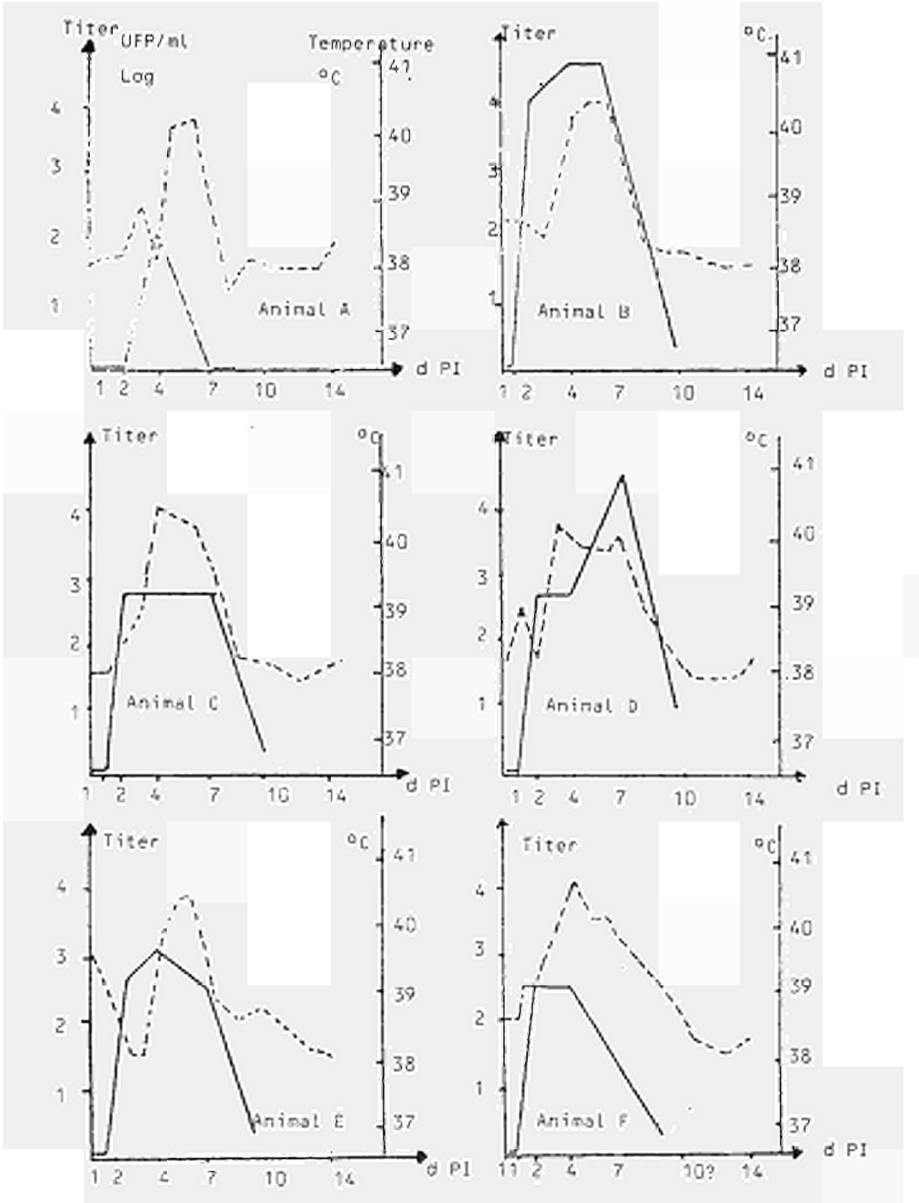
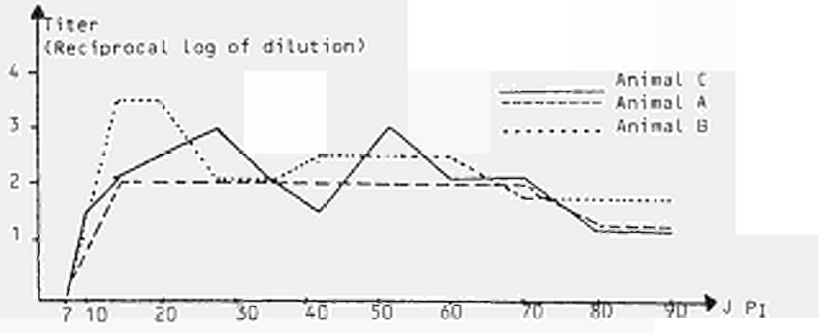


Fig. 1 : Virus excretion in trachea and rectal temperatures BHV-1 experimentally infected heifers.



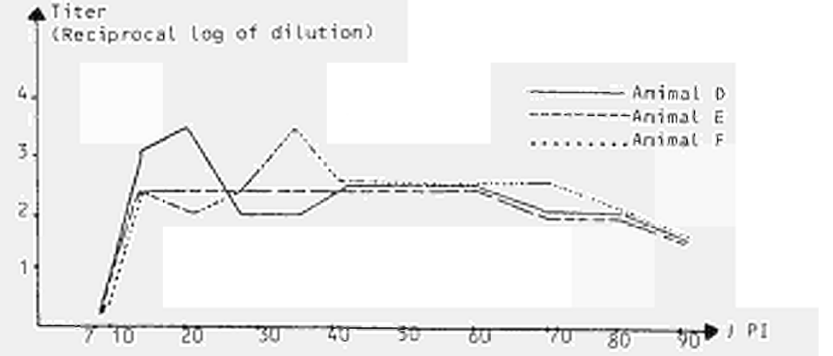


Fig. 2: Neutralizing antibodies in BHV-1 experimentally infected heifers.

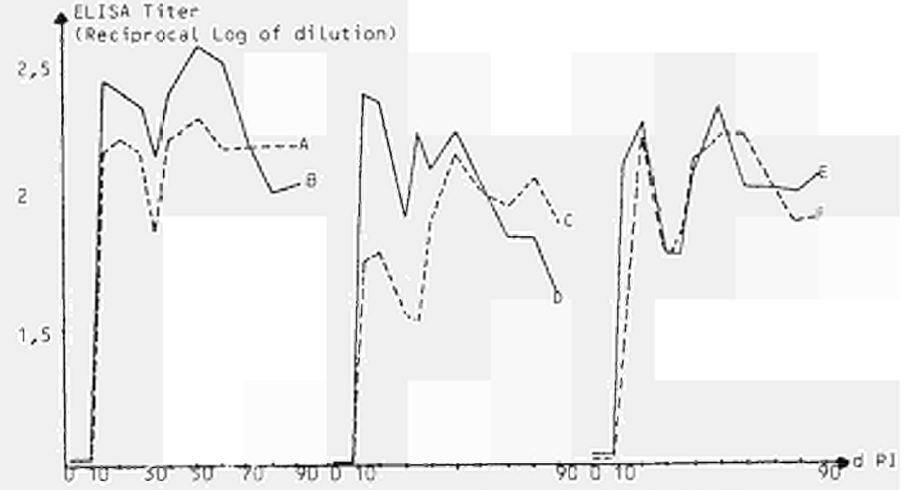
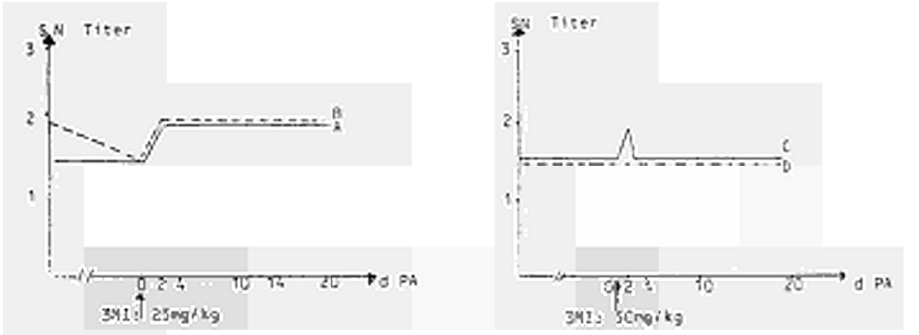
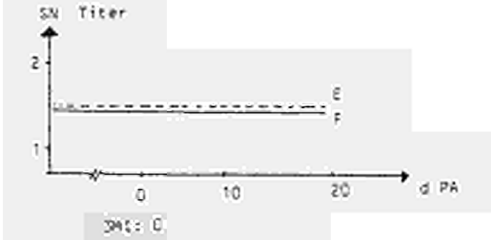
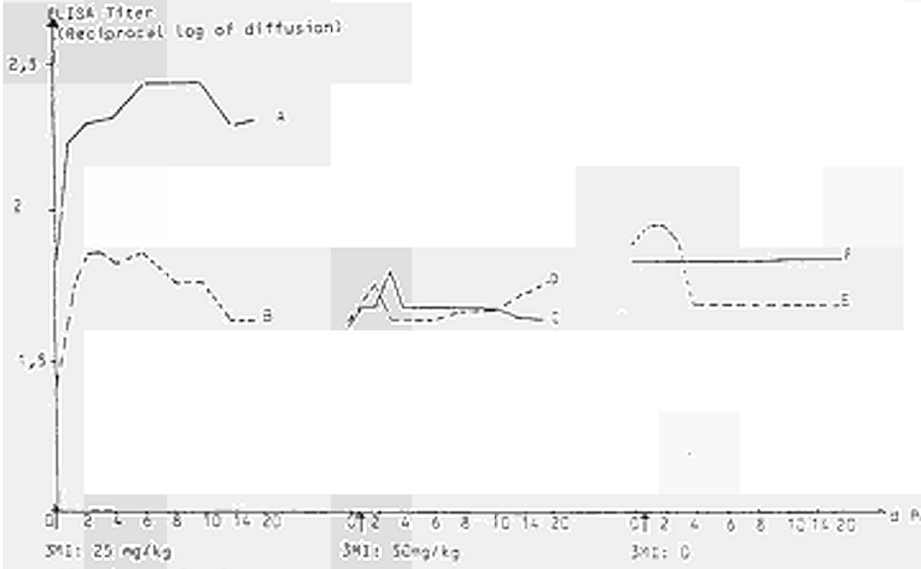


Fig. 3: ELISA antibodies in BHV-1 experimentally infected heifers.





a) Neutralizing antibodies.



b) ELISA antibodies;

Fig. 4: Serological findings after the first 3 MI administration.

Bovine Herpesvirus 1 life vaccine causes persistent infection of the genital tract with spontaneous shedding of virus in the semen.

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ABSTRACT

Bulls inoculated with BHV-1 life vaccine shed virus with semen up to several months after vaccination. Characterization of the viral DNA by restriction enzyme analysis revealed that DNAs of isolates were indistinguishable from DNA of the vaccine strain. These results emphasize the need for a safe efficacious and compatible BHV-1 vaccine.

INTRODUCTION

The virus responsible for Infectious Bovine Rhinotracheitis/Infectious Pustular Vulvovaginitis (IBR/IPV) infections, classified as Bovine Herpesvirus type 1 (BHV-1) is spread worldwide (Ludwig, 1983). Like other herpesviruses it causes latent infections, from which virus can be reactivated by natural or artificial stress factors. Humoral antibodies do not prevent virus latency but have an influcence on the frequency of recurrent infections and on the amount of excreted virus (Pastoret et al., 1980).

Being aware of virus latency, inactivated BHV-1 vaccines have been tested for their ability to control the spreading of IBR-infections, but life virus vaccines appeared to be more efficacious (Straub, 1978). During the last years modified life BHV-1 vaccines have been favoured speculating that vaccine virus would occupy places of viral latency and thus prevent the field virus to become latent (Kretschmar, 1981). In contradiction excretion of vaccine virus and field virus after vaccination and subsequent challenge with field virus was observed (Sheffy and Rodman, 1973, Nettelton and Sharp, 1980, Nettelton et al., 1984).

We report on the excretion of BHV-1 identified as vaccine virus which occured in bulls of an artificial insemination center.

MATERIALS AND METHODS

Vaccination

A commercial "IBR/IPV-Vakzine" was given to 25 breeding bulls in two artificial insemination (AI) centers. One dose was applied intraprepucially, another dose was inoculated in equal quantities into the nostrils. 3 weeks later and in intervals of several months up to three booster inoculations were given.

Virus isolation

Without prior immunosuppressive treatment of the animals semen of the bulls and washings from the inner wall of the artificial vagina were taken and used for virus isolation. After incubation of embryonal calf lung cells with the inoculum, cultures were observed for cytopathic changes during a maximum of three passages. Mock infected, parallel cultures did not contain BHV-1 specific antigen when assayed by immunofluorescence tests. Isolated virus was identified by virus neutralization using a defined BHV-1 antiserum from an hyperimmunized rabbit.

Serum neutralization test

Neutralization test were performed as microtitre method in a constant virus (100 $TCID_{50}$) varying serum system. Antibody titres are given as highest serum dilution causing total virus neturalization.

Restriction enzyme analyses

Viral DNA was isolated according to a standard method (Pignatti et al., 1979) from infected Georgia bovine kidney cells after 2-4 passages. DNA was digested with the following restriction endonucleases: BamHI, BstEII, EcoRI, HindIII, HpaI, KpnI, SstII according to the manufacturer's (BRL) instructions. Fragments were separated in 0.7% agarose gels, stained with ethidium bromide (Sigma, 1 mg/1000 ml H_2O) and visualized in UV-light (306 nm).

RESULTS

Virus could be isolated from the semen of 6 out of 25 bulls. Preputial washings of three of the 6 animals contained virus, too. The intervals between the last vaccination and virus isolations were 60 or 81 days, respectively. Virus isolation was also successfully isolated from two nitrogen frozen semen samples of one of the virus shedding animals (Table 1).

Case No.	NT-AB[1]	BHV-1 life virus vaccinations	NT-AB[2]	BHV-1-isolation from semen	BHV-1-isolation from prepuce	days after last booster
1	neg.	2	8	+		60
				+ L.N.	+	
				+		
2	neg.	2	8	+	-	60
3	neg.	3	2	+	+	81
4	neg.	3	8	+	-	60
5	neg.	4	2	+	+	81
6	neg.	4	4	+	-	60

TABLE 1: Vaccination history and subsequent virus isolation from breeding bulls.

1) BHV-1-neutralizing antibodies before vaccination.
2) BHV-1-neutralizing antibodies at the time of viurs isolation/reciprocal serum dilution.
L.N.: Liquid nitrogen frozen semen samples.

DISCUSSION

Our finding of an uncontrolled shedding of BHV-1 vaccine virus several months after inoculation without any stimulation by corticosteroids should be considered for further vaccination strategies. Especially the excretion of vaccine virus with the semen of breeding bulls and the possibility of its wide spreading after conservation in liquid nitrogen causes serious problems.

The genital excretion of virus suggests, that vaccine virus had established and replicated in the testicles or in the spermatic ducts without being inactivated by the immune response. This interpretation is supported by the finding that from 6 animals excreting virus with the semen only 3 had virus on the preputial mucosa. Furthermore, the finding of of BHV-1 antigen in the sperm heads by EHLAZHARY et al. (1980) becomes noteworthy in this context.

Although our observations cover only a relatively short observation period the results show that major genome alterations are not detectable after animal passage. Presumably no infection with field virus had occurred. Before immunization all the animals were free of BHV-1-specific antibodies and there is little chance of a later infection under the conditions in an AI-center.

There are good reasons to differentiate BHV-1 life virus vaccine strains derived from isolates of IPV-cases and those descending from IBR infections. Abortions after life virus vaccination with strains causing the death of the fetuses or later on of the calves have been reported (McFeely et al., 1968; Kelling et al., 1973; Mitchell, 1974; Darcel le Q. and Dorward, 1975; Pastoret et al., 1980).

These reports came from North America, where IBR virus strains prevail and were commonly used as vaccine virus. There are no hints that similar events occurred in Germany where the vaccines are made of attenuated IPV-virus strains. Interestingly enough, however, virus of the IBR-type has been recovered on independent occasions from spleen and other tissues of healthy fetuses (Ludwig and Storz, 1973; Storz et al., 1980). From earlier investigations we know that abortogenic BHV-1 isolates represent IBR viruses whereas no abortogenic IPV-virus strain is known (Gregersen, 1983; Pauli et al., 1984). These findings are supported by the fact that abortion has never been described in connection with typical IPV-virus infections (Pauli, 1981; Ludwig, 1983).

The choice of an IPV virus as vaccine strain obviously eliminates the risk of vaccine-induced abortion. Nevertheless such a vaccine does not fulfill the requirements for a safe vaccine because it leads to latent infections, can be excreted late after vaccination and can therefore be spread uncontrolled. These findings emphasize the urgent need for an efficacious, compatible and safe BHV-1 vaccine.

After these results had been obtained, no further vaccination was performed. One year later virus isolation was tried again, but turned out to be negative.

By restriction enzyme analysis with six different enzymes we investigated the genomes of the isolates in order to identify them as vaccine or field virus strains. Furthermore we used the HpaI fragment pattern for the differentiation of IBR from IPV strains (Pauli et al., 1984, Gregersen et al., in press). The results were as follows: All isolates showed identical DNA fragment patterns. The genome profiles of the isolates were identical to those of the vaccine strain used, suggesting that all isolates represented re-isolates of the vaccine strains. Figure 1 shows as an example the restriction enzyme patterns of isolates, vaccine strain, one IPV- and one IBR-strain after EcoRI digestion.

Vaccine strain and isolates clearly differ from IBR virus strains and were identified as IPV viruses according to their HpaI fragment pattern.

Fig. 1 EcoRI restriction enzyme pattern of the DNA from isolates, vaccine virus, IPV and IBR-virus.

Lane 1-8: DNA of the eight BHV-1 isolates from vaccinated bulls.

Lane 9: DNA of the virus used for vaccination.

Lane 10: DNA of an IPV-virus and lane 11: DNA of an IBR-virus.

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IMMUNOLOGICAL STUDIES DURING LATENT FELID HERPESVIRUS 1 INFECTION

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ABSTRACT

Felid herpesvirus 1 (FHV) causes a severe upper respiratory tract disease in cats. There are only limited reports of immunity during latent FHV infection and these mostly relate to serum neutralising antibody. The association of FHV-specific responses (serum neutralising antibody, cytotoxic antibody and lymphocyte transformation) and non-specific immune function (Con A lymphocyte transformation) with natural, "stress"-induced recurrence of latent FHV infection was investigated. Immunosuppression was not associated with induction of virus shedding, although the cats which shed had a lower baseline cytotoxic antibody capacity, underwent a more severe "stress" response to a change of housing and had initially experienced a more severe primary disease episode than the cats which did not shed. A marked elevation in activity of specific immune function was associated with the occurrence of shedding.

INTRODUCTION

Felid herpesvirus 1 causes a severe upper respiratory tract disease of felidae. In common with specific herpesvirus infections of many other species, the primary infection is followed by a latent infection in the host which may periodically reactivate with shedding of infectious virus. Following FHV 1 infection, at least 82% of cats have been identified as carriers by induction of virus-shedding episodes (Gaskell and Povey, 1977). Such shedding was shown to occur spontaneously, following corticosteroid immunosuppression, or after the "natural"

physiological "stress" of a change of housing (up to 1%, 69% and 18% of a group of cats respectively). The role of the immune system in the control of latent herpesvirus infections has been subject to much investigation prompted by observations of more frequent and/or more severe recrudescent disease in patients with immunodeficiency syndromes or undergoing immunosuppressive therapy (Reviewed by Babiuk and Rouse, 1979). Such studies have examined potential differences in immunity between individuals which were susceptible to frequent recurrent herpesvirus disease and non-susceptible individuals and have attempted to detect immune deficits associated with the induction of recrudescence.

Studies on human patients, however, were confounded by clinical and ethical considerations, and this was further emphasised by studies of immune function during recrudescence, where sequential studies were severely limited by difficulty in prediction of such episodes. Experimental animal models of human herpesvirus recurrence involved physical trauma or pharmacological interference (Openshaw et al, 1981; Klein, 1982), which did not represent "natural" stimuli. Similarly, studies of induced recurrence in domestic animals have utilised immunosuppression (Turner et al, 1970; Davies and Carmichael, 1973; Gaskell and Povey, 1977; Pastoret et al, 1979; Wittman et al, 1983). For the study of immune events potentially involved in recurrence, a non-immunological inducer is obviously to be preferred, and the ability to provoke such episodes by rehousing "stress" in the cat provides a natural means of achieving this in the natural host-herpesvirus system. This paper describes immune events associated with susceptibility to, and induction of virus shedding. The study has been reported in detail elsewhere (Goddard, 1984; Goddard et al, manuscript in preparation).

MATERIALS AND METHODS

Cats

Seventeen specific pathogen free-derived cats were rehoused from communal controlled environment housing into individual external isolation pens.

Cats were monitored daily for clinical signs and virus shedding for 7 days prior to and 21 days after rehousing. Prior to rehousing blood samples were assayed on five occasions for total and differential leukocyte counts, FHV-specific and Con A lymphocyte transformation (LT) response, and on two occasions for serum neutralising antibody titres (SNT) and antibody-complement lytic antibody capacity (CP). After rehousing blood was sampled on alternate days for 21 days and then at weekly intervals for 2 weeks.

Virus Isolation

Oropharyngeal and nasal swab samples were inoculated onto confluent monolayer cultures, using standard techniques, and examined for FHV cytopathic effects.

Clinical Scores

Upper respiratory tract disease signs were scored from 0 (normal) to 5 (severe) to give a total daily score. A "stress score" was recorded as clinical score for inappetance plus scores for behavioural disturbance (1 = nervous, 2 = overtly scared, 3 = physical withdrawal). Leukocyte Counts were estimated by standard laboratory techniques.

Serum Neutralising Antibody Titres (SNT)

A microassay adaptation of the method of Povey and Johnson (1969a and b) was used, and results expressed as reciprocal $\log_2$ serum dilution. Changes more than 2 $\log_2$ were regarded as significant.

Antibody-Complement lytic capacity (CP)

A standard ^{51}Cr release microassay described previously (Goddard et al, 1984) was used. The % specific lysis of serum at $\log_4$ dilutions of 1, 2 and 3 was recorded, arcsine transformed, and the area under the dilution curve

calculated to give the lytic capacity.

Lymphocyte transformation (LT)

FHV-specific LT and Con A induced LT were measured using the whole blood microassay system described previously (Goddard et al, 1984). Results were expressed as net (test-control) quench-corrected dpm.

The pre-rehousing parameters of each cat were used to calculate upper and lower significance limits ($P < 0.05$) for that cat to assess change after rehousing. Non-parametric measurements were analysed by the Mann-Whitney U Test.

RESULTS

A detailed report of these results is given elsewhere (Goddard et al, manuscript in preparation).

Virus isolation

Three of 17 (17.65%) cats (1, 2 and 3) shed infectious FHV on post rehousing days 4-12, 7-14 and 8-10 respectively.

These cats had undergone a significantly more severe primary disease episode than the cats which did not shed ($P = 0.036$).

The results of cat 3 were different to those of the 2 other shedders and were considered to have been influenced by contact with another shedding cat 15 days before rehousing. This was 23 days before its own shedding period and was regarded as a separate episode.

Immune Functions

(1) The pre-rehousing Period.

The mean resting CP of the three shedder cats was significantly lower ($P < 0.1$) than that of the non-shedder cats. No other significant differences were observed.

(2) The Post-rehousing Period.

The three shedder cats had a significantly greater mean "stress" response to rehousing ($P=0.025$) than the non-shedder cats. No significant depression of any parameter was observed after rehousing in the shedder cats. However, isolated significant reductions were seen in some

of the non-shedder cats, particularly in one cat which had a selective lymphopoenia on post-rehousing day 1 associated with a significant depression in Con A LT and the most marked stress response in the experiment.

(3) The Post Shedding Period.

Shedder cats 1 and 2 showed marked boosting of SNT, CP and FHV-LT commencing during, and continuing after the virus shedding period. FHV-LT was still rising at 40 days post-rehousing, whereas SNT and CP responses reached a plateau at 20-30 days. Shedder cat 3 had much less marked responses at the time of shedding, and there was evidence of some specific boosting prior to rehousing, which could have resulted from the suspected contact infection.

DISCUSSION

In these studies, using a "natural" stimulus to induce recrudescence, no significant suppression of specific or non-specific immunity was associated with the induction of FHV shedding, and in fact the only significant reductions observed were in non-shedder cats, which may well argue against an absolute role for immunosuppression in induction of shedding. There have been few reports of immune events prior to natural herpesvirus recrudescence in other systems, but recently subtle changes in lymphokine production and suppressor lymphocytes have been reported (Sheridan et al, 1982). Thus in the present study such changes may have occurred undetected.

The increase of FHV-specific parameters during the active shedding period observed in 2/3 shedders in the present study was consistent with reports of recrudescent human herpesvirus disease where most described either no change (Rasmussen et al, 1974; Sheridan et al, 1982) or an increase (Shillitoe et al, 1977 and 1978; Rattray et al, 1980) in specific LT. While reports of LT were variable, the most consistent changes during this period related to raised lymphokine and interferon production (Rosenberg et

al, 1974; O'Reilly et al, 1977; Reichmann et al, 1977; Rattray et al, 1980; Sorrensen et al, 1980; Sheridan et al, 1982). The discrepancies between parameters might be explained by the hypothesis that immune effector functions (e.g. cytotoxicity, lymphokines) are more important in herpesvirus control than memory functions (e.g. LT response) (Sheridan et al, 1982).

The consequences of shedding in this study were boosting of FHV-specific responses. In other systems increases in serum antibody titres have been reported (Sorrensen et al, 1980; Sekizawa et al, 1980) although most workers reported no change (Rasmussen et al, 1974; Lopez and O'Reilly, 1977; Shillitoe et al, 1977 and 1978). However, the results were consistent with those reported after FHV recrudescence by Gaskell and Povey (1977). HSV-specific LT has been observed to decline after recrudescent episodes (Shillitoe et al, 1977 and 1978; Rattray et al, 1980) in contrast to the more persistent elevation of FHV-specific LT seen in the present study. Again, in HSV systems it was lymphokine and interferon secretion which manifested marked change (increase) after recrudescence (Rasmussen et al, 1974; Shillitoe et al, 1977 and 1978; Cunningham and Merigan, 1983) and this is currently under investigation for FHV in the cat.

The different response of shedder cat 3 was thought to have resulted from the contact infection experienced prior to rehousing. Such observations may be relevant to the effect of vaccination on carrier cats.

The cats which shed virus had a significantly lower base-line cytotoxic antibody capacity, greater "stress" response to rehousing and had undergone a more severe primary disease episode than non-shedder cats. Reports of differences between herpes simplex virus (HSV) sero-positive shedders and non-shedders in man have frequently given equivocal results. However, a rise of in vitro interferon secretion after recrudescent HSV infection has been found to correlate well with the time to the next

episode (Rasmussen et al, 1974; Cunningham and Merigan, 1983). In addition to reduced lymphokine secretion (Wilton et al, 1972; Gange et al, 1975; Donnenberg et al, 1980; Sheridan et al, 1982), other "effector functions" - specific direct lymphocyte cytotoxicity (Steele et al, 1975; Thong et al, 1975; Fujimiya et al, 1978) and non-specific cytotoxicity (Wilton et al, 1972) Antibody dependent cytotoxicity (Fujimiya et al, 1978) and neutrophil mobility (Rabson et al, 1977) have also been shown to be depressed in susceptible patients. In contrast most reports found that the susceptible individuals had higher serum antibody levels, although the results of work on herpes-specific LT is equivocal (Steele et al, 1975; Russell et al, 1976; O'Reilly et al, 1977; El Araby et al, 1978). The interaction between stress and immune function is being increasingly studied (Kelley, 1980) and the"stress" response to rehousing may have affected the immune response of the cats, to facilitate overt virus shedding.

Reductions in the immune functions measured did not appear to be fundamental in induction of recurrent FHV shedding, although some significant differences in parameters, behaviour and disease history of shedders and non-shedders were observed. However, regardless of the actual signal for reactivation of latent virus, it is the integrative activity of the immune system which controls the overt shedding of infectious virus and thus studies of herpesvirus immunity are crucial for the understanding of these diseases. This "natural" system provides a very useful means for the study of a herpesvirus in its natural host. Further studies with this system, which avoids pharmacological or physical interference with the host animal, may clarify the role of immunity in induction of, and susceptibility to herpesvirus shedding.

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SUMMARY OF SESSION FIVE.

HERPESVIRUS IMMUNITY AND REACTIVATION.

by P.-P. PASTORET.

The introductory paper given by T.J. HILL (United Kingdom) described the mechanisms underlying the establishment, maintenance and reactivation of latent herpesvirus infections. This paper deals mainly with the mouse model of infection with human herpesvirus (Herpes simplex virus). This model is relevant for natural herpesvirus infections of domestic animals, since it allows the carrying out of numerous experiments to clarify the underlying mechanisms. From these studies three main conclusions may be drawn:

- immune mechanisms may prevent the access of the virus to the target cells in which latency is established;
- when latency is established, the immune responses do not seem to be important for its maintenance;
- following reactivation, immune mechanisms are likely to be involved in the clearance of the virus from the tissues.

In the veterianry field, the phenomenon of re-excretion is of great importance, from the epidemiological point of view. In this respect, it is important to better our understanding of the conditions necessary for herpesvirus reactivation. A paper presented by E. THIRY (Belgium) states that reactivation of Bovine herpesvirus 1 (BHV 1) is induced by several stimuli, such as transport or Dictyocaulus viviparus infestation. Another paper from ESPINASSE and co-workers (France) indicates that, from immunological evidence, reactivation of BHV 1 seems to be induced also by the administration of 3-Methylindole, a powerful pulmonary toxic.

The latency of attenuated vaccine strains used in the veterinary field is another aspect of the problem as seen from the practical side. A paper presented by GREGERSEN (West Germany) shows that bulls vaccinated with BHV 1 live vaccine shed virus in the semen for up to several months after vaccination. It is concluded that further studies are still required in order to produce new vaccines that will be as efficacious as the existing attenuated vaccines but completely devoid of side effects such as latency; such vaccines will help control vaccine virus dissemination.

Finally, a paper presented by GODDARD (United Kingdom) deals with the immunological behaviour of cats latently infected by Felid herpesvirus 1. The study shows a behaviour similar to that of other herpesviruses already studied. A marked elevation in specific immune function was associated with the occurrence of a shedding episode.

SESSION 6

NOVEL APPROACH FOR THE PREVENTION OF HERPESVIRUS INFECTIONS

Chairman : H. LUDWIG

Co-chairman : L. THIRY

APPROACHES TO VACCINATION AGAINST HERPESVIRUSES: FROM ATTENUATION OF VIRUSES TO RECOMBINANT AND SYNTHETIC SUBUNIT VIRUS VACCINES

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INTRODUCTION

The herpesviruses constitute a virus group that, at the end of infection, take refuge in the host's nervous system and become latent. Reactivation of the latent herpesvirus leads to a new episode of disease, in spite of the previous exposure of the immune system to the virus, and in spite of the presence of neutralizing antibodies. In immune-compromised hosts, herpesvirus infections can become life-threatening. Thus, the immune system in its response to the viral antigens plays an important role in reducing the severity of the infection caused by the herpesvirus and probably enhances the defences against further recurrencies of the virus. However, this does not prevent the production of the virus in individuals who carry a reactivable latent herpesvirus and its spread to individuals who have not previously been exposed to the virus.

Various approaches to the research and development of herpesvirus vaccines for humans and animals are summarized in Table 1. However, due to the unique properties of herpesviruses, it has been impossible to develop vaccines against this virus for use in man. The ability of herpes simplex virus (HSV) types 1 and 2 to transform cells under in-vitro conditions (Duff and Rapp, 1971) has so far prevented its use for the development of an attenuated live virus vaccine.

The use of a live attenuated vaccine against a highly pathogenic tumor-inducing herpesvirus of chickens (Marek's disease virus; MDV) was achieved by Churchill and his colleagues (Biggs et al., 1970). Today, chickens are immunized with a naturally occurring herpesvirus of turkeys (HVT) (Witter et al., 1970; Nazerian, 1979). The chickens are inoculated with a cell-associated live virus which prevents the development of lymphoid tumors when infection with an oncogenic MDV occurs. Introduction of the HVT live virus vaccine markedly reduced economic losses for the chicken industry and proved

TABLE 1 Approaches to development of live and subunit herpesvirus vaccines

1. Live attenuated viruses

   Selection of naturally occurring viral mutants;

   selection of virus mutants or mutants with a deletion in the virulence genes

2. Subunit virus vaccines

   Cloning of viral glycoprotein genes in bacterial plasmids;

   expression and synthesis of HSV-1 gD (and other glycoproteins) in E. coli, yeast, or mammalian cells;

   purification of viral gD for use as a subunit virus vaccine

3. Synthetic peptides

   Amino acid sequences of the antigenic epitopes can be synthesized and used as viral antigen in a vaccine

4. Live recombinant viruses

   Gene(s) for the herpesvirus antigens can be cloned in an attenuated virus (poxvirus or another attenuated herpesvirus) which will be used as a live virus vaccine

to be an effective way to prevent Marek's disease. However, the mechanism by which HVT protects chickens against MDV is still unknown.

The use of attenuated pseudorabies virus strains for the immunization of pigs against Aujeszky's disease is now a common practice (Wittmann and Hall, 1982). Immunized pigs do not develop the disease after infection with a virulent wild type pseudorabies strain, but the virus can become latent in infected pigs and, when reactivated, the virulent virus can spread to, and infect, other pigs (Wittman et al., 1984). Studies on pseudorabies vaccine strains revealed that the thymidine kinase-negative (TK^-) viruses had reduced virulence (Lomniczi et al., 1983). Extensive research on HSV-1 has shown that the thymidine kinase (TK) gene plays an important role in the virulence of the virus (Field and Wildy, 1978; Tenser et al., 1981; Gordon et al., 1983; Ben-Hur et al., 1983, Becker et al., 1984a, b). Elimination of the viral TK gene from the virulent virus genome is not enough if a completely avirulent virus is needed; the neurovirulence gene (Table 2) must also be inactivated or deleted from the virus genome. It is also necessary to remove DNA sequences which might be involved in cell transformation (Jariwalla et al., 1980; Galloway et al., 1982).

Cloning of genes for HSV-1 glycoproteins in bacterial plasmids and their expression in bacteria, yeast, or mammalian cells provide a new approach to the development of herpesvirus subunit vaccines. These pro-

TABLE 2 Herpes simplex virus-1 genes involved in neurovirulence to mice

Herpesvirus virulence genes	Map position in virus genome	Attenuation of virus	Role in virus infection
Thymidine kinase	0.291-0.316	Mutations in promoter sequence and in the gene sequence	Replication in neurons and CNS
Neurovirulence	0.760-0.787	Deletion	Replication in brain (Thompson et al., 1983; Rösen et al., in press)

cedures allow for large-scale industrial production of viral glycoproteins which can be used for immunization of man or animals (Long et al., 1984). The viral antigens produced by this biotechnological approach, as well as synthetic peptides which contain the antigenic epitopes of the glycoprotein D of herpes simplex virus type 1 (Cohen et al., 1984) can be used to elicit antibody responses in the vertebrate host, which can neutralize both HSV-1 and HSV-2.

Another approach (Table 1) is the development of recombinant poxvirus vaccines into which the HSV-1 TK gene is introduced (Panicali and Paoletti, 1982). Herpesvirus glycoprotein genes could also be introduced into such recombinant viruses,which would confer immunity against the poxvirus as well as the herpesvirus (Mackett et al., 1984).

In this presentation, I will discuss some of the approaches used in my laboratory to study the attenuation of HSV-1 by selection of mutants, the analysis of viral glycoproteins of HSV-1 isolates, the cloning of the HSV-1 viral glycoprotein genes, and attempts to obtain their expression in E. coli and mammalian cells.

MATERIALS AND METHODS

Viruses and cell cultures

The TK mutants used in this study were originally selected on the basis of plaque size from the NIH strain of HSV-1 that has a mixed plaque morphology of large and small plaques. The NIH wild type (wt) virus strain No. 11124 was propagated in BSC-1 cell monolayers grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 10 per cent calf serum. From the wt virus that produced a heterogeneous population of large (2 mm diameter) and small (0.5 mm diameter) plaques, we obtained a large plaque

(LP) TK^+ isolate, a small plaque (SP) $TK^{1/4}$ mutant with 25 per cent of the thymidine kinase activity of the LP TK^+ virus, and an LP TK^- mutant that did not express TK at all (Gordon et al., 1983; Ben-Hur et al., 1983).

Pathogenicity of virus mutants in mice

Outbred albino mice of various ages were infected with the HSV-1 strains by inoculation onto scarified corneas (under ether anesthesia) at a concentration of 10^6 or 10^7 pfu/ml. A drop (50-100 µl) of virus suspension was placed on each cornea, and the eyes were rubbed. The mice were observed for three weeks, and virus pathogenicity was determined by the number of mice that died.

Four-week-old mice were susceptible to infection with the LP TK^+ isolate of wt virus that replicated in the trigeminal ganglia and brain, and killed most of the mice. The TK^- mutant virus with no TK activity did not infect the trigeminal ganglia or kill the mice. The SP $TK^{1/4}$ virus strain replicated in the eyes within 24 hr after inoculation and entered the trigeminal ganglia, establishing a latent infection in almost all the mice, but did not reach the brain or kill the mice (Gordon et al., 1983).

Cloning of HSV-1 genes in pBR322

The Q fragment (BamHI-BamHI) containing the TK gene from HSV-1 strain F was cloned in pBR322 to yield plasmid pBRTK. In plasmids pBY16 and pBY53-12, the TK gene was taken from the HSV-1 LP TK^+ and SP $TK^{1/4}$ isolates, respectively (Becker et al., 1984a,b). In plasmid pBY40-2, the BglII/I DNA fragment of HSV-1 LP TK^+ was cloned in the BamHI site of pBR322.

Plasmid pBY718/pBY7(17) was derived from the BglII-BamHI sequence of the Q fragment of HSV-1 strain F cloned in pBR322. This plasmid also contained the BamHI J fragment of the viral DNA of LP TK^+ (Becker et al., 1984a,b) with the genes for glycoprotein D (gD) and part of gE. Plasmid pBY40-2 contained the BglII/I DNA fragment of the HSV-1 LP TK^+ strain with the genes for the DNA binding protein (DNA BP), glycoprotein B (gB) and glycoprotein A (gA).

Plasmid pHABY119 contained the promoter left (P_L) and N gene (N) from phage λ DNA originally present in pHA10 (Honigman, 1981; Becker et al., 1983). The BglII-BamHI DNA fragment from HSV-1 LP TK^+ containing the HSV-1 TK gene was cloned in the BamHI site. Plasmid pHABY130 was derived from pHABY119 by cloning a SacI-SacI DNA fragment carrying the HSV-1 gD gene in the SacI site of the HSV-1 TK DNA.

Precipitation of HSV-1 glycoproteins from BSC-1 cells with monoclonal antibodies

BSC-1 cells were infected with HSV-1, and after 6 hr the cells were labeled with ^{35}S-methionine (sp. act. 1400 C/mmol, Amersham Int., England). At 20 hr post-infection, the cells were rinsed three times with PBS and dispersed in 300 µl of extraction buffer consisting of 1% NP-40, 0.1 mM phenylmethyl sulphonylfluoride (PMSF) in PBS pH 8.0 at 4°C for 15 min. After centrifugation at 15,000g for 15 min, 20 µl of monoclonal antibodies were added to 100 µl of supernatant fluid. This was kept at 4°C overnight and then 50 µl of Protein A-Sepharose Cl-4B beads (40% v/v, Pharmacia, Sweden) in extraction buffer was added for 1 hr at 4°C. The beads were washed three times with extraction buffer and 50 µl of electrophoresis buffer (2% SDS, 5% mercaptoethanol, 10 mM Tris-HCl, pH 7.0, 10% sucrose and 2 mg/ml bromophenol blue) was added. After boiling for 2 min and centrifugation, the supernatant fluid (50 µl) was placed on 10% acrylamide gels.

Neutralization of HSV by monoclonal antibodies directed against gB

Virus suspensions were incubated with monoclonal antibody B5 (obtained from Prof. M. Levine, The University of Michigan, Ann Arbor, Michigan) for 2 hr at room temperature. The virus was titrated on BSC-1 monolayers.

Expression of HSV-1 gD gene in E. coli

Plasmids pHABY119 and pHABY130 were transfected into E. coli strain 157 (C600 λcI857) (obtained from Prof. H. Engelberg-Kulka, Dept. of Molecular Biology, our medical school) and grown overnight in LB medium containing ampicillin at 30°C. After dilution to 40 Klett units (blue filter) in medium M9 containing 1% casamino acids, the bacteria were grown at 30°C to 80 Klett units and then transferred to M9 medium containing an amino acid mixture without methionine and $MgSO_4$. Each batch was divided into two and incubated for 2 hr at 30°C and 42°C, respectively. Then 0.1 ml from each flask was transferred to an Eppendorf tube to which was added 10 µCi of ^{35}S-methionine (sp. act. 1400 C/mmol; Amersham Int., England). The tubes underwent stationary incubation for 90 min at either 30°C or 42°C. The bacteria were washed with PBS and 100 µl of a solution of 2 mg lysozyme,and 1 ml PBS was added for 10 min at 4°C. After centrifugation, 100 µl supernatant fluid was mixed with 20 µl monoclonal antibodies to gD (supplied by Prof. Myron Levine, University of Michigan, Ann Arbor,Michigan). This was kept at 4°C overnight, mixed with Protein A-Sepharose beads and

prepared for electrophoresis in 10% acrylamide gels as described above.

RESULTS

Mutations in the HSV-1 TK gene lead to attenuation of virulence

The relationship between the viral TK gene and virulence is presented in the following experiments (Ben-Hur et al., 1983). Infection of mice at different ages with the virulent virus strain (TK^+) by inoculation onto scarified eyes resulted in the development of encephalitis and over 90% of the infected mice died (Fig. 1). Both virus mutants (TK^- and $TK^1/_3$) had lost

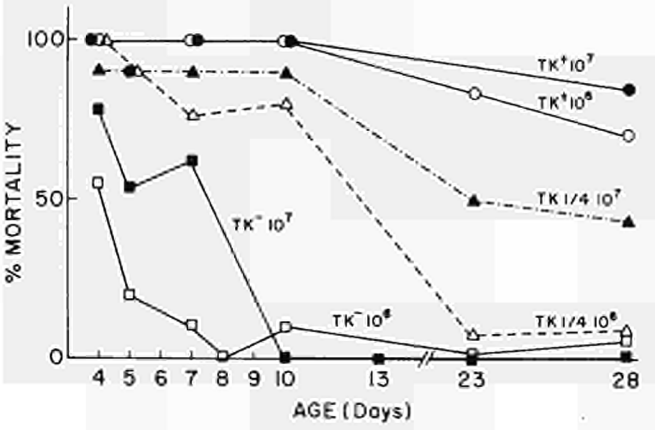


Fig. 1 Pathogenicity of HSV-1 virus strains in mice of different ages. The LP TK^+, SP $TK^1/_3$ and LP TK^- isolates were inoculated onto scarified corneas at inoculum doses of 10^6 and 10^7 pfu/ml. (Reprinted with permission from Ben-Hur et al., 1983).

most of their virulence for four-week-old mice. However, the infecting dose is also a determining factor: 10^7 pfu/ml of $TK^1/_3$ virus was virulent, whereas 10^6 pfu/ml was not. An additional factor is the age of the mice: the TK^- mutant was virulent for young mice during the first week of their life (Fig. 1), but 10-day-old mice infected with it were not killed. The $TK^1/_3$ virus isolate, which is less pathogenic than the TK^+ virus, demonstrates the development of gradual resistance to HSV-1 infection with increasing age. Mice were sensitive to the $TK^1/_3$ virus up to ten days of age, after which their sensitivity gradually decreased.

TABLE 3 Possible sites for mutations in the HSV-1 TK gene leading to resistance of the virus to TK-activated antiviral drugs

1. Mutations in the gene sequence
   a. Deletion of nucleotides
   b. Point mutations
2. Mutations in the regulatory sequences of the TK gene
   a. Mutations in nucleotides in the RNA polymerase binding site
   b. Mutations in the CCCCGCCC or GGGGCGGC sequences in the α protein binding site (expression enhancer)
   c. Mutations in the TATA box
   d. Mutations in the palindromic sequence (attenuator sequence) centrally located between the mRNA start and the ATG codon of the TK gene

Nature of mutations in the HSV-1 TK gene which lead to attenuation

The promoter sequence of the HSV-1 TK gene with its functional domains is presented in Fig. 2 (Becker et al., 1984b). It is possible that a mutation in one of the functional domains in the promoter (Table 3) will affect the level of gene expression in infected cells. Thus, a mutation in the TK gene at the active site will lead to synthesis of inactive TK enzyme molecules, and the resulting virus strain will be TK⁻ (Becker et al., 1984a,b).

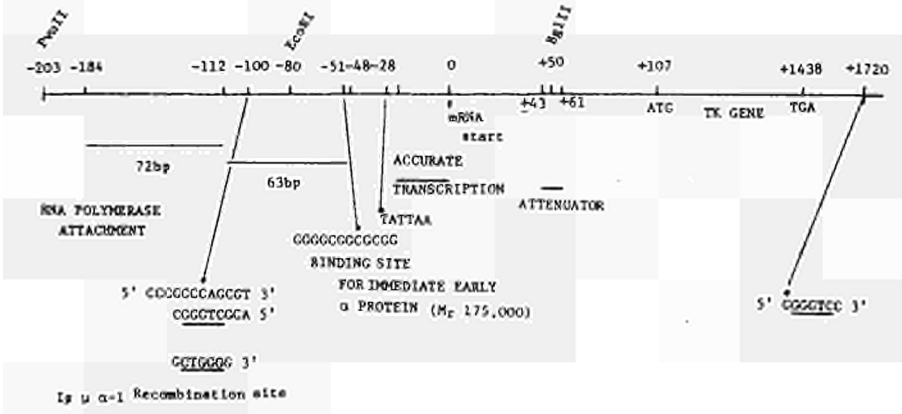


Fig. 2 A schematic organization of the HSV-1 TK gene promoter with its functional domains. (Reprinted with permission from Becker et al., 1984b).

TABLE 4. Mutations in functional domains of HSV-1 TK promoter elucidated from sequencing of EcoRI-HpaII fragments

EcoRI-HpaII sequence isolated from plasmid	Level of TK activity	Functional promoter sites: -100 (Recombination)	-50 α protein binding site	0 Accurate transcription	+50 Attenuator	+107 ATG	+121 HpaII site
pBRTK[a]	TK^+	NC	NC	NC	NC	NC	MUTATION C → T
pBY16[b]	TK^+ (lower than pBRTK)	NC	"MUTATION"	"MUTATION"	NC	NC	NC
pBY53-12[c]	TK^-_4	NC	NC	"MUTATION"	"MUTATION"	NC	NC

NC = No change

[a] pBRTK = The TK gene (BamHI-BamHI) from HSV-1 strain F cloned in pBR322

[b] pBY16 = as in pBRTK, but TK gene cloned from HSV-1 LP TK^+ strain

[c] pBY53-12 = as in pBRTK, but TK gene cloned from HSV-1 SP TK^-_4 (Becker et al., 1984a,b)

The EcoRI-HpaII fragment spanning the BglII site was obtained from each TK gene, and the nucleotide sequence was determined. The mutation of the HpaII site was a base change (C → T). "Mutation" indicates breakage of the DNA during hydrolysis and indicates a possible change in nucleotides leading to DNA fragmentation.

Such TK^- mutants can be selected by treating infected cells with bromodeoxyuridine (BUdR). The virus particles that survive are the TK^- mutants, and these have been shown to have lost their pathogenicity for mice as described above.

Another type of mutation is the production of expression mutants that have a lower level of TK expression (e.g. TK^-_4 mutant). The mutation may have occurred in the attenuator sequence in the promoter (Fig. 2; Becker et al., 1984b), most likely in the sequence for accurate transcription. As a result of this mutation, the TK^-_4 virus produces TK at a level of 25% in infected LTK^- mouse cells, as compared with the TK^+ virus. Reduction in the expression of the TK gene in the TK^-_4 mutant resulted in attenuation of the infectivity of this virus strain in four-week-old mice (Fig. 1). However, the TK^-_4 mutant did not lose its pathogenicity for young mice. Sequencing of the EcoRI-HpaII fragments in the nucleotide sequence of the TK genes cloned from a number of HSV-1 isolates revealed the actual sites of mutation (Table 4).

TK^- mutants that can easily be isolated may be defective not only in the TK gene but also in the functional domains of the TK promoter sequence.

Such mutants differ from each other in the degree of TK gene expression and, therefore, in their neurovirulence for young and for four-week-old mice.

Plaque size of mutants and the TK gene

An additional number of mutants was selected on the basis of plaque size from large plaques (2-4 mm in diameter) and from small plaques (0.2-0.5 mm in diameter). From each isolate, we prepared a TK^- mutant, using BUdR (the parent strain was TK^+). The pathogenicity of these strains for mice was studied by inoculation of the virus onto scarified corneas of four-week-old mice. Table 5 shows that the small plaque (SP) mutant 11 (designated SP11 TK^+), which had an active TK gene, was less virulent (80.7% survivors) than either the LP TK^+ isolate (2.8% survivors) or the SP18 TK^+ isolate (5.4%). This result indicates that in the SP11 TK^+ isolate an unknown gene was mutated which led to reduction in virus pathogenicity. A TK^- mutant isolated from SP11 TK^+ (designated SP11 TK^-) had lost its virulence completely and was not pathogenic for mice by the eye route even at 10^7 pfu/ml (T. Ben-Hur and Y. Becker, to be published).

The virus isolate, SP18 TK^+, was found to be highly virulent for mice, even after selection of a TK^- mutant (SP18 TK^-; Table 5). These observations indicate the presence of a neurovirulence gene in addition to the viral TK gene, as indicated in Table 2.

The LP and SP isolates can be identified by restriction endonuclease analysis of the viral DNA (Fig. 3). LP isolates (lanes 3 and 5) have an additional band between the J(16Kb) and K(11Kb) fragments. Another extra band is present between the K and L fragments. These extra bands are not present in the SP, F and Justine strains of HSV-1.

TABLE 5 Virulence of HSV-1 isolates in mice inoculated in the eye

Virus isolate	Surviving mice	%
LP TK^+	1/36	2.8
LP TK^-	65/65	100.0
SP11 TK^+	21/37	80.7
SP11 TK^-	10/10	100.0
SP18 TK^+	2/37	5.4
SP18 TK^-	40/55	72.7

Mice were infected by corneal scarification with 10^7 pfu/ml of virus, and their survival was determined over a three week period.

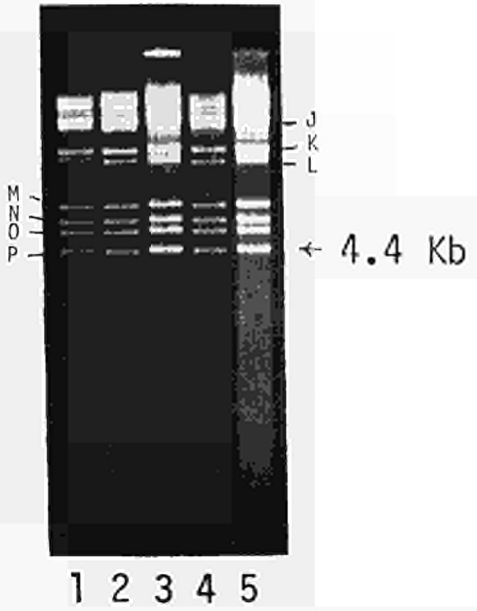


Fig. 3 BglII restriction enzyme analysis of HSV-1 strains:

1) F, 2) SP TK½, 3) LP TK⁺, 4) Justine, 5) LP TK⁻

The DNA preparations were electrophoresed in 0.7% agarose containing ethidium bromide.

Analysis of viral glycoproteins in the plaque size mutants using monoclonal antibodies

Glycoproteins gD and gB produced in BSC-1 cells infected with the four virus strains: LP TK⁺, SP TK½, and the two small plaque mutants SP11 TK⁺ and SP18 TK⁺ were compared (Fig. 4). The amounts of gD and gB glycoproteins precipitated by specific monoclonal antibodies (generously provided by Prof. M. Levine) were similar in the plaque size mutants tested. Glycoprotein C was also produced to the same extent by the four virus strains (not shown). Thus, no differences were found in glycoproteins among the various plaque size isolates. Nonetheless, it was of interest to determine whether monoclonal antibodies to gB can neutralize virus infectivity in these isolates. As shown in Table 6, infectivity was reduced by the anti-gB monoclonal antibodies in the LP TK⁺, SP TK½, and SP18 TK⁺ isolates, but SP11 TK⁺ was not affected at all. This result indicates that the antigenic epitope of gB on the outer membrane of SP11 TK⁺ virions was not detectable by the monoclonal antibody. It is not known if this particular missing gB epitope is associated with the decrease in pathogenicity of this virus.

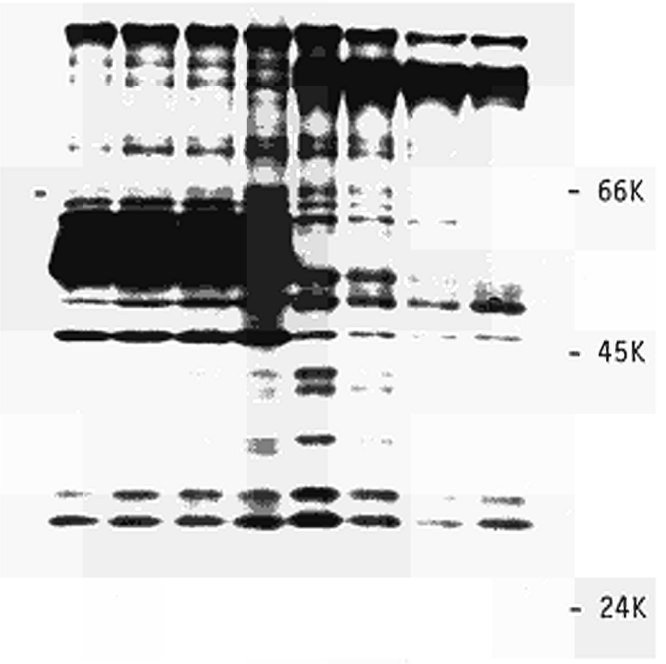
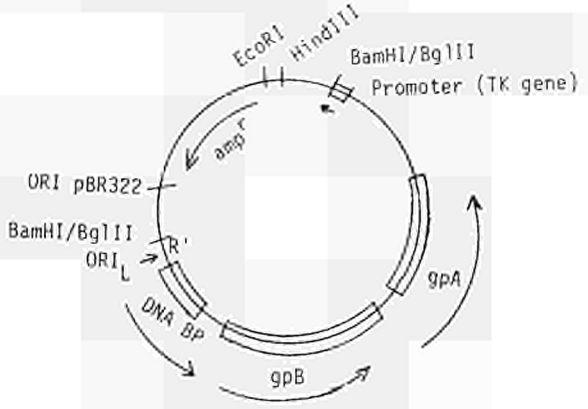


Fig. 4 Precipitation of HSV-1 isolates with monoclonal antibodies to gD and gB. The virus strains were grown in BSC-1 cells and treated with monoclonal antibodies to gD and gB as described in Materials and Methods. Samples were electrophoresed in 10% acrylamide gels.

TABLE 6 Neutralization of HSV-1 mutants by plaque reduction with monoclonal antibodies to glycoprotein B

Serum dilutions	LP TK$^+$		SP TK$^-$		SP11 TK$^+$		SP18 TK$^+$	
	Exp 1	Exp2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
	pfu/plate							
Control	150	100	119	186	200	360	120	320
1:50	89	--	92	--	200	--	55	--
1:100	86	67	72	125	200	360	52	230
1:200		65		120		360		240
1:400		68		125		360		160
1:800		58		110		360		160

Monoclonal antibodies to HSV-1 glycoprotein B (monoclonal B5 obtained from Prof. M. Levine, The University of Michigan, Ann Arbor, Michigan) were diluted and added to each virus suspension. Bovine calf serum was used as control. The suspensions were incubated at room temperature for 2 hr before plating on BSC-1 plates. After a 2 hr adsorption period, the plates were covered with agar and incubated at 37°C for 3 days. The agar was removed, and the cells were stained with 0.1% gentian violet. The average number of plaques per two plates was determined.



pBY40-2

Fig. 5 A diagrammatic presentation of recombinant plasmid pBY40-2. The BglII/I DNA fragment of HSV-1 LP TK$^+$ strain was cloned in the BamHI site of pBR322. The orientation of three HSV-1 genes is indicated: DNA binding protein (DNA BP), glycoprotein B (gpB) and glycoprotein A (gpA). The promoter of the viral TK gene is present in the cloned DNA fragment.

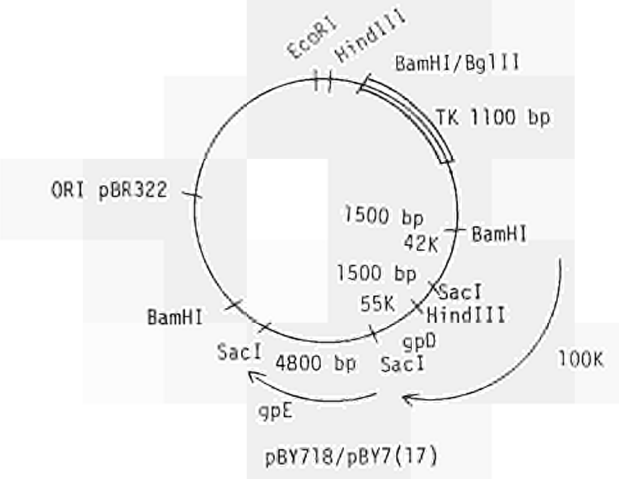


Fig. 6 A schematic representation of the recombinant plasmid pBY718/pBY7(17). This plasmid contains the BglII-BamHI sequence with the HSV-1 TK gene. Two additional viral genes of HSV-1 strain F (gD and gE) were cloned in the BamHI site of pBY718. In the resulting recombinant plasmid, an unknown viral gene is present upstream to the viral gD gene. The viral gE gene is downstream to the gpD. The orientation of the viral genes is indicated by the arrows.

Cloning and expression of HSV-1 glycoprotein genes in transfected L(TK$^-$) cells

Two plasmids containing the gB/gA and the gD glycoprotein genes in pBR322, respectively (pBY40-2, Fig. 5; pBY718/pBY7(17), Fig. 6) were used to transfect LTK$^-$ cells using the HSV-1 TK gene for the selection of TK$^+$ transformants. From the transfected LTK$^-$ cells (Becker et al., 1984b), individual colonies were obtained and propagated in HAT medium. The colonies which were designated with the number of the recombinant plasmid used for transformation were studied for the expression of the viral genes. The gB/gA genes were not expressed in the cell colonies transformed by plasmid pBY40-2, as determined by the use of monoclonal antibodies to precipitate ^{35}S-methionine-labeled proteins. This might be due to the inability of the cellular RNA polymerase II to recognize and transcribe HSV-1 gamma genes which require the attachment of a beta gene protein product to its promoter (Roizman, 1983).

Analysis of the ^{35}S-labeled proteins of the colony transformed with plasmid pBY718/pBY7(17) revealed a 100K polypeptide which was precipitated by monoclonal antibodies to HSV-1 gD. The gD polypeptide has a molecular

weight of 55K (Morse et al., 1978; Lee et al., 1982). It is possible, however, that the 100K polypeptide recognized by the gD monoclonal antibodies is a fusion product, namely a polypeptide initiated in the gene which codes for a 42K polypeptide. This was described by Lee et al. (1982) and precedes the gD gene in the HSV-1 genome. The 42K gene is a beta gene which can be recognized by the cellular RNA polymerase II. Further studies are in progress to characterize this protein.

Expression in E. coli

The DNA fragment carrying the gD gene was cloned in the SacI site of the HSV-1 TK gene which was inserted downstream from the N gene of the λ phage left promoter under the control of the cI^{ts} repressor (cI857). The resulting plasmid was designated pHABY130 (Fig. 7). This plasmid was introduced into E. coli strain 157 (C600λcI857) and the transfected bacteria were incubated at 30°C and 42°C. As a control, E. coli strain 157 was transfected with plasmid pHABY119 and incubated at 42°C. The bacterial homogenates were treated with monoclonal antibodies to gD and the antigen-antibody complexes were precipitated and analyzed by polyacrylamide gel electrophoresis. Fig. 8 shows that, in the absence of monoclonal antibodies

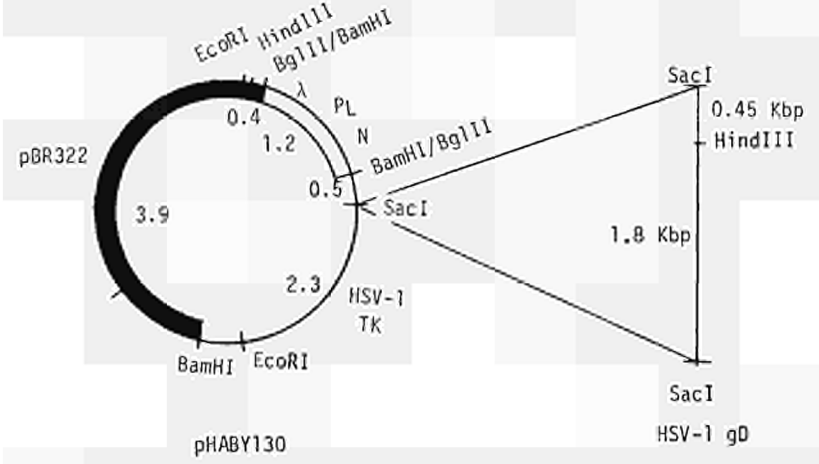


Fig. 7 A schematic representation of plasmid pHABY130. Plasmid contains the left promoter (P_L) and N gene (N) from λ DNA. The BglII-BamHI HSV-1 LP TK^+ DNA fragment containing the HSV-1 TK gene was cloned at the BamHI site. A SacI-SacI DNA fragment which carries the HSV-1 gD gene was cloned in the SacI site in HSV-1 TK DNA.

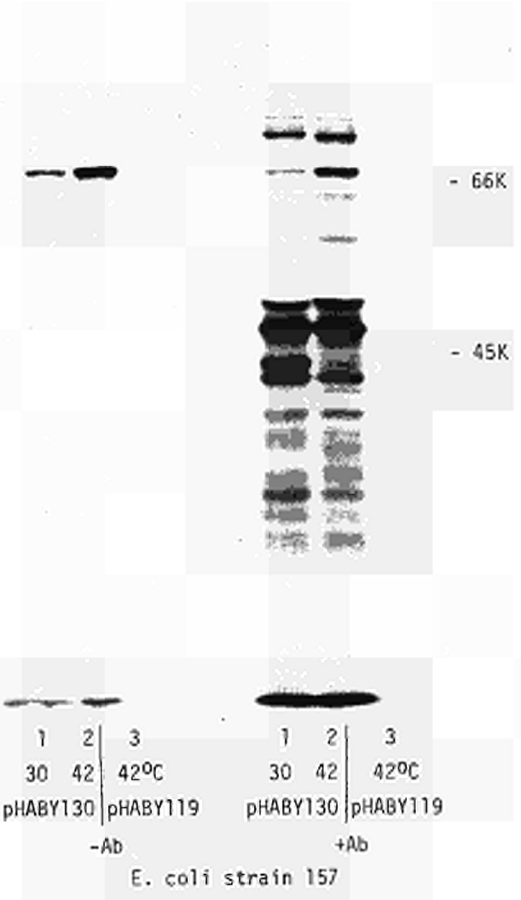


Fig. 8 Expression of HSV-1 gD gene in E. coli. Plasmid pHABY130 was incubated at 30 and 42°C and plasmid pHABY119 at 42°C, after which monoclonal antibodies to gD were added, as described in Materials and Methods. Samples with and without gD were electrophoresed in 10% acrylamide gels.

only one polypeptide band (∿66K) was obtained. In samples treated with gD monoclonal antibodies, proteins were precipitated from bacteria carrying plasmid pHABY130 at 30 and 42°C. The nature of these proteins is being studied, as is the question as to whether or not they are related to gD. It has been demonstrated that HSV-1 gD produced in E. coli is antigenic and can protect mice against challenge with a virulent strain of HSV-1 (Weis et al., 1983).

DISCUSSION

The development of new technologies of gene transfer has made it possible not only to identify viral genes involved in neurovirulence and malignancy, but also to use these genes for the production of viral antigens for vaccination purposes. Gene transfer technology has also made it feasible to transfer genes from different viruses into poxviruses, which can then be used for vaccination of humans, animals and chickens against a number of diseases in addition to those caused by poxviruses. In this way, the naturally occurring attenuated poxvirus vaccine, which was introduced into medicine by Jenner in 1796, can be utilized as a carrier for the genes of hepatitis B virus, influenza virus, or herpes simplex virus (Smith et al., 1983; Mackett et al., 1984). New and exciting possibilities thus exist for the use of engineered recombinant live virus vaccines for immunization of sensitive humans or animals against pathogenic viruses.

The availability of recombinant live virus vaccines does not preclude the need for herpesvirus live attenuated vaccines. Immunization of the human eye against local infection with herpes simplex virus might reduce the incidence of herpes keratitis in humans. An attenuated live herpes simplex virus vaccine needs to be engineered in such a way that the genes coding for neurovirulence (TK and neurovirulence genes) will be mutated or deleted, in addition to deletion of the nucleotide sequences involved in cell transformation.

Table 7 documents the response of cells in the reticuloendothelial system (RES) to live or killed viruses, as well as to viral proteins or peptides in synthetic and natural virus subunit vaccines. The attenuated live viruses involve the RES in immunizing the host and lead to life-long immunity against the virus. It is possible that the subunit virus vaccines will be useful in eliciting neutralizing antibodies to herpesvirus glycoproteins (Cohen et al., 1984). However, the live herpesvirus and recombinant poxvirus vaccines will also succeed in eliciting local tissue and RES immunity. Live attenuated vaccines like those used to protect chickens and pigs could be developed for herpesviruses of other domestic animals with the help of genetic engineering technologies.

Table 7 Involvement of the reticuloendothelial system in the immune response to live-attenuated or killed virus vaccines

Type of vaccine	Site of inoculation	Fate of virus or viral antigen	Reticuloendothelial element involved	Immune response
Attenuated virus	Epithelial layers (e.g. skin: scratch or intradermal)	Replication in epithelial cells	Langerhans cells	Virus replication leads to survival of Langerhans cells and presentation of antigens to the lymphocytes.
Killed virus particles	Intramuscular Intraperitoneal	Enter blood circulation. Taken up by macrophages	Kupffer cells (liver). Dendritic cells (spleen)	Virus particles are taken up by the reticuloendothelial cells and viral antigens are presented to the lymphocytes.
Viral proteins or peptides	Intramuscular	Enter blood circulation	Kupffer cells (liver). Dendritic cells (spleen)	Low uptake? Low level of presentation of viral antigen to lymphocytes?

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VACCINATION OF HUMANS AGAINST VARICELLA-ZOSTER VIRUS

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ABSTRACT

Clinical trials with live vaccines prepared with the attenuated Varicella-Zoster Virus (VZV) OKA strain indicate that this vaccine induces humoral and cell-mediated responses in healthy and in immunocompromised individuals. In a proportion of the latter individuals, the vaccine may cause mild varicella or zoster but it affords a 100% protection against severe disease after contact. There is no indication that vaccination of healthy adults harbouring latent VZV will protect against VZV reactivation and zoster symptoms.

INTRODUCTION

This paper tries to underline the principal features of the meeting which took place in Munchen on the 29th-30th November 1984. The author hopes that she did not inadvertently introduce some bias in the presentation of the lively discussions of the meeting. A full paper of this meeting will be published in a book.

The need

There is hardly any symptomless case of varicella. Chicken pox is as inevitable as death and taxes. Rates of encephalitis complications per 100,000 cases are 1.7 and 15 in children and adults respectively, as well as 3.2 of Reye syndrome (N.B. The latter figure may vary with countries, since Belgian pediatricians refuted Reye syndrome association with chicken pox ; the highest rate of this syndrome is certainly registered during influenza epidemics). The world wide annual number of Zoster is estimated at 5.2×10^6, with the cruciating pain which this disease involves, and the non negligable proportion of motor deficit sequellae.

In immunocompromised patients, very severe varicella or Zoster were observed in 11% of children with lymphoma, 30% of those with leukaemia (7 % of the leukemic children died of VZV infection) and in as much as 50% of bone marrow transplanted patients. In one german study of 109 children with acute lymphocytic leukaemia, there was a 2% mortality and 19% morbidity due to VZV (7 varicella, 11 Zoster, 1 encephalitis and 1 pneumonia). However,

these figures will be now strikingly reduced with the introduction of the highly effective acyclovir therapy.

There are some discrepancies in the figures relating to the risk of congenital varicella. Gisela Enders in Germany followed women who did not possess VZV antibodies at the beginning of pregnancy ; among those with seroconversions, 4% only gave rise to abnormal infants. According to this author VZV intrauterine infection differs from rubella in that infection of the foetus does not lead to permanent virus production in the tissues, with no antibodies of the IgA and IgM class detected in the newborn. There is however a prolonged high titer of CF IgG. Distribution of the risks was quoted by Stanley Plotkin. Eighteen women with VZV symptoms during prenancy (15 with varicella, 3 with Zoster) gave birth to abnormal children. Nine of these women showed symptoms during the first trimester, 8 during the second and 1 during the third trimester. Ten infants had typical limb deformities, 13 had ocular abnormalities, 9 suffered from central nervous system involvment and 10 showed varicella cicatrices.

The problem

Varicella Zoster Virus (VZV) being a member of the herpesgroup, there is a theoretical risk that the vaccine might cause cancerous transformation. Most of us harbour wild VZV in a latent state but we do not know how much it contributes to our risk of cancer. Transformation might be more likely with an attenuated strain, if one refers to works with Herpes Simplex Virus which does not cause transformation when fully cytopathic, but only acquires transforming properties after partial inactivation. VZV present in the attenuated vaccine is the OKA strain developed by doctor Takahashi and extensive research has shown that cells were not transformed by this strain. In Japan, there is already a ten year experience with the vaccine but one may argue that this delay is not yet sufficient, on the one hand, and that, on the other hand, negative attempts at in vitro transformation do not reflect the in vivo situation. In Japan, the complications of chicken pox have been estimated to heavily outweight the potential risk of remote cancer and generalization of the vaccination is recommended, although not with a rigid protocol.

On the other hand, the risk that VZV vaccine strain may establish in the latent form is real and will be discussed below.

From the point of view of the vaccine producer, VZV is uneasy to handle because it is cell associated with low yield in the supernatant and a low

ratio of intracellular virus to cell proteins. This implies tedious purification procedures and high cost of vaccine.

The immunogen

Monoclonal antibodies to wild type VZV glycoprotein recognize three groups of epitopes. Site A migrates with a 105,000 MW protein and is a target for neutralizing activity of antibodies. Site B epitope is found in 5 distinct polypeptide bands of 98 to 45,000 MW, some of which are probably precursors of the others. These sites are targets for complement dependent neutralizing activity. (The actual total amount of neutralizing activity in an individual must be assayed in the presence of complement).
Finally, epitopes of site C migrate with two proteins of 66 and 57,000 MW and are not targets for neutralizing activities.
To our knowledge, the epitopes present in the vaccine strains are not yet well documented.

The immune response

Humoral antibodies are best evaluated by an immunofluorescence test (FAMA) which detects membrane associated viral antigens. It is recommended to introduce an internal control, i.e. to mix an excess of non infected cells with the VZV producing cells. This test equals in the evaluation of neutralizing antibodies. Elisa are somewhat less reliable but are prefered for large scale studies.

In vitro evaluation of cell-mediated immunity by lymphocyte transformations assays is less reliable than an in vivo skin test. However, there is a great need for a standardized antigen.
Frequent local reinfections do occur after the natural disease. This is reflected by frequent boosters at the level of secretory IgA antibodies. At long interval periods, some of the reinfections probably penetrate further within the organism, since boosters at the level of serum IgG antibodies and cell-mediated responses were noted after interval periods of decades.

The importance of cell-mediated immunity for protection against the disease is proven. The issues of close contact which chicken pox cases were followed in individuals with a low titer of FAMA antibodies (1:8 and 1:16). Of those with a negative skin test, 6% contracted varicella symptoms, while no symptoms occurred in individuals with a positive skin test. Another study showed that all of the individuals with a positive skin test have neutralizing antibodies, while 6% of those with the antibodies do not show

positive skin tests. Vaccination of the latter individuals induced the appearance of cell-mediated response.

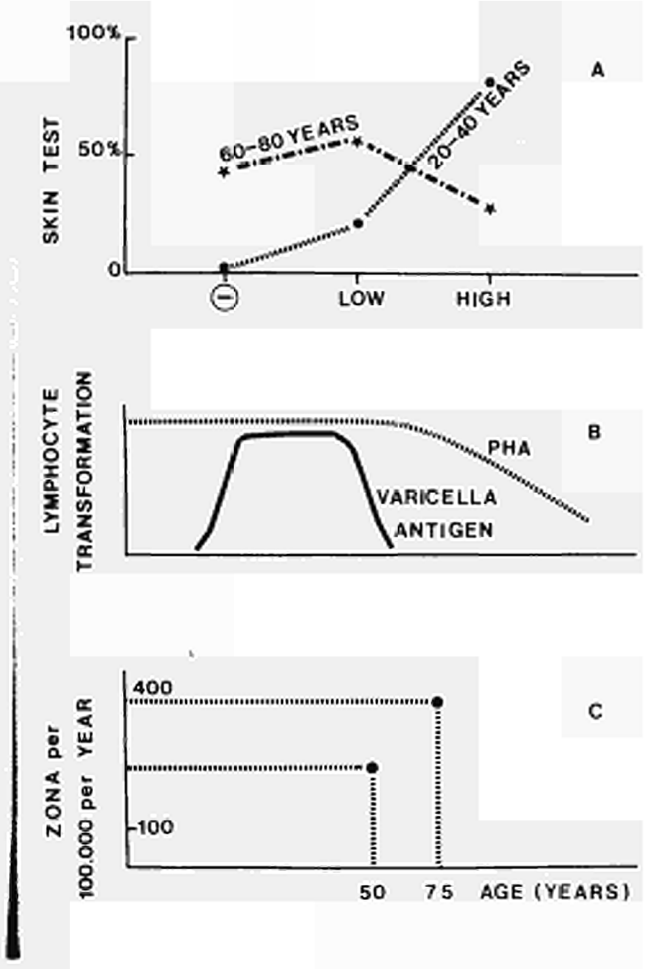

Fig. 1 Correlation between frequency of zoster and loss of cell-mediated immunity to VZV. A : Proportion of individuals whith high (large diameter), low (small diameter) or no (-) skin reaction to VZV antigen. B : Decrease with age of lymphocyte response to phytohaemagglutinin and to VZV. C : Proportion of individuals with Zoster symptoms according to age.

Figure 1 shows that the number of individuals with a positive skin test to VZV antigen strikingly decreased with age. In vitro, non specific lymphocyte response to phytohaemagglutinin decreases more slowly than that to VZV antigen. The age at which cell-mediated response to VZV wanes corresponds to the striking increase of Zoster incidence. If we live long enough, 15% of us will suffer from Zoster symptoms.

Vaccination : efficacy and complications

Inoculation of 5000 PFU of VZV vaccine will elicit antibody formation in 90 to 100% of children, as well as cell-mediated response. Infants respond well but seronegative adults appear to be less receptive to the vaccine, and a higher dose than 5000 PFU may be needed. High degree of infant susceptibility and relative resistance of adults remind of the situation with hepatitis B vaccine.

After vaccination, humoral antibody level seems to decrease somewhat more rapidly than would be expected from a live vaccine, but positive skin tests are still observed many years after vaccination. Nevertheless, usefulnes of a booster dose of vaccine is envisaged.

In contrast to natural disease, vaccination does not induce local secretory IgA nor does it increase natural K cell activity.

In immunocompromised patients, vaccination also provokes a high seroconversion rate as well as good cell-mediated response. This is probably due to the fact that VZV vaccine strain is more invasive in these patients than in healthy individuals. The increased amount of viral antigens produced may compensate for the low level of immune response capacity. However, viral vaccine invasion results in the appearance of mild symptoms in some individuals of this group. Although it is recognized that VZV vaccine will afford a 100% protection efficacity against severe varicella, all the study groups showed a frequency of mild rash and/or Zoster within a short period after vaccination. The worst reactions are noted in leukemic children vaccinated during a relapse and without interruption of the antileukemic treatment. Up to 55% of these children developed a mild rash post vaccination and 7% suffered from a not severe zoster. This indicates that the vaccinal strains may establish within the neutral ganglia and there are a few examples that this can even occur in healthy individuals with no immunosuppression background. Post-vaccination mild rashes may be contagious to close contacts and thus vaccinated leukemic children must be kept away from other people

at risk. Figures for the post vaccinal complications are greatly lowered if the leukaemic patient is vaccinated while in remission or at a moment when lymphocyte counts reach at least 1200 per ml, or if antileukaemic treatment is suspended for at least one week before and one week after vaccination.

In patients with lymphoma, solid tumours or steroid treatment, clinical symptoms are induced by the vaccine in 5%, 9.6% and 2.7%, respectively.

Vaccination versus specific immunoglobulins or acyclovir treatment

Before introduction of vaccination and active drugs, specific immunoglobulins directed at VZV were efficiently used to prevent or decrease varicella symptoms, if the immunoglobulins were injected within a few days after a contact. However, since contact risks are high among children, immunoglobulins often had to be injected as frequently as every 3 months. Moreover, many contacts occurred of course without notice. On the other hand, passive immunisation with immunoglobulin injections does not imply suspensions of antileukaemic treatments in contrast to acyclovir administration or vaccination. Vaccination however presents the great advantage that the moment of treatment interruption may be appropriately chosen.

Discomfort for the patient must also be taken into account. In this respect, acyclovir treatment perhaps ranks the worst, since three daily perfusions of several hours each are necessary.

Long term effects of the three treatments are being followed. Several studies indicate that anti VZV vaccination does not increase the risks of leukaemia relapses, and that this probably holds true for acyclovir administration. As stated above, vaccine may cause mild Zoster early after vaccination, but does not increase the long term risk of Zoster due to wild type virus reactivation. Actually, some studies seem to indicate that vaccination has some beneficial influence on future natural zoster. Wild type and attenuated VZV isolated from Zoster vesicles can be readily differenciated by restriction enzymes (endonuclease) mapping.

STRATEGIES

A. Minimal

A "coup par coup" policy will aim at identifying persons at risk and providing a vaccination at the least unfavourable moment. Decreasing prio-

rities rank as follows :

- Leukaemic children
- Tumour patients, especially children
- Children with cystic fibrosis (Pediatricians notice that varicella as well as measles not only are severe diseases in these patients, but that they can dramatically precipitate the course of cystic fibrosis)
- Uremic patients (especially children) prior to kidney transplantation
- Seronegative adults (or better, adults with a negative skin test) with a priority to health personal who represent a possible link for a chain of VZV transmission, as well as to child bearing age women,with contraindication of vaccination during pregnancy. Hazards of live VZV vaccinations for the foetus have however not been proven.

B. Maximal (optimal ?) : the social barrier policy

It is realised that the above scheme represents a heavy procedure not likely to be worlwidely applied. Attempts at long term eradication of VZV is thus envisaged. M. Just in Switzerland has applied the following vaccination scheme to 15 month old children. On the same day, these children received 7 different vaccines

1 - MMR (live measles, mumps, rubella) in one seringe
2 - VZV vaccine in a second seringe
3 - DT (diphteria, tetanos toxoids) in a third seringe
4 - Sabin polio vaccine per os

Post vaccination antibodies were of the same order of magnitude than in cases of separate vaccinations. However, mixing VZV with MMR seemed to reduce antibody response. Other workers obtained good results with a mixture of MMR-VZV. It was agreed that the appropriate optimal amounts of MMR-VZV antigens in a mixture have still to be defined.

Cost benefit analysis of course demonstrated a striking benefit for this combined vaccination policy. In particular, generalization of VZV vaccination would decrease the cost per dose, since quality controls for large batches are always more economical.

If the above policy were applied with patience, tenacity and the will for win, a social barrier to the viruses would insure a double protection for the individuals at risk. No only would these individuals meet the wild viruses much more rarely, but the social barrier would enable the high risk groups to escape the hazards of vaccination : immunocompromised patients

might be spared VZV vaccination while whildren prone to convulsions would be spared measles vaccination and childbearing age women would receive booster rather than primary rubella vaccination.

ON THE IMMUNITY INDUCED IN PIGS BY CONVENTIONAL VACCINES AND DELETION MUTANTS OF AUJESZKY'S DISEASE VIRUS.

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ABSTRACT

Vaccination of pigs against Aujeszky's disease (AD) is common practice in many countries. In general, attenuated AD virus vaccines are more efficacious than inactivated vaccines. In addition, they can be given intranasally and this mode of vaccination induces better protection than parenteral vaccination(s). However, none of the present AD vaccines is capable of conferring complete protection against a challenge with a virulent virus.

Attenuated AD viruses used as vaccines are usually obtained by numerous passages in cell culture. Consequently, (i) in these strains mutations are introduced in an uncontrolled way, and (ii) most attenuated strains contain several variants. The above observations indicate a need for more potent and better defined AD virus vaccines.

Several approaches are possible for the development of new AD virus vaccines. Because (i) attenuated vaccines are basically more efficacious and (ii) the observation that pigs which survive an infection with virulent AD virus are fully protected against challenge 6 weeks later, we have chosen for bio-engineering a "live" AD virus vaccine, starting from a virulent and highly immunogenic AD wild-type virus. We have constructed hybrid viruses of a virulent and an avirulent strain of AD virus and subsequently assayed for virulence in mice and pigs. A conclusion from this study was that the Us region comprises virulence genes. Subsequently, deletions were introduced in the Us region of the genome of the virulent strain. The deletion mutants generated were strongly reduced in virulence, and were highly immunogenic in pigs.

INTRODUCTION

Aujeszky's disease (AD) has a worldwide distribution. The pig is the natural host of AD virus and the only significant source of virus spread. In many countries vaccination of pigs is practised to reduce severe economical losses. Attenuated (live) as well as inactivated (killed) vaccines are available. Because comparative data on efficacy of AD vaccines are scarce, we have compared efficacy of the vaccines most frequently used in the Netherlands in sero-negative pigs under standardized laboratory conditions. In the Netherlands, most breeding pigs are vaccinated against AD twice a year and as a result young pigs acquire maternally derived antibody (MDA). It is difficult to achieve an effective immunization of pigs

with MDA. The first results of intranasal (IN) vaccination of pigs with MDA were encouraging (De Leeuw et al., 1982) and subsequently we compared the efficacy of IN vaccination with an attenuated AD virus with parenteral vaccination(s) with inactivated vaccines.

On several grounds the presently used attenuated AD virus vaccines are susceptible of improvement. In recent years, DNA recombinant technology has become available that may be employed to construct well-characterized, safe and effective "live" AD virus vaccines.

The present report deals with the results of comparative studies on attenuated and inactivated AD virus vaccines, and with the construction and testing of deletion mutants of AD virus.

Comparison of efficacy of AD virus vaccines in sero-negative pigs

We have compared 2 inactivated vaccines (A= Nobivac®, Intervet, the Netherlands, B= Geskyvac®, Roger Bellon, France) and 4 attenuated vaccines (C= MK-25®(strain MK-25), Pharmachim, Bulgaria, D= Ay-vak®(strain B-KAL), Pliva, Yugoslavia, E= Delsuvac®(strain BUK/TK 650), Gist-Brocades, the Netherlands, F= an experimental vaccine (strain Bartha) provided by Duphar, the Netherlands).

Two vaccines (C en D) were found to be excreted after vaccination.

Table 1 summarizes the results on the efficacy of the 6 vaccines. Despite a maximum of standardization, considerable variation still existed in mortality and growth arrest of control pigs after challenge. All vaccines, except vaccine E prevented mortality after challenge, but none conferred complete protection. Most vaccinated pigs still lost weight, developed fever and shed virus after challenge. The results of this comparative study indicate that 2 attenuated vaccines (D and F) conferred the best protection, that 1 attenuated vaccine (C) was of equal efficacy as the 2 inactivated vaccines (A and B) and the 4th attenuated vaccine (E) was the least effective.

TABLE 1. Summarized results of standard tests to evaluate efficacy of AD virus vaccines.

Vaccine	Mean SN-titer at challenge	Challenge results			
		No. dead / No. tested	Mean days of		
			Growth arrest	Fever*	Virus shedding
A	100	0/6	8	5	⩾10
A 2x	500	0/7	6	5	8
Contr.	-	2/7	16	7	⩾10
B	30	0/8	7	4	6
B 2x	300	0/8	5.5	5	5
Contr.	-	3/8	24.5	8	8
C	100	0/8	6	3	4
C 2x	250	0/7	6.5	3	5
Contr.	-	4/7	17	6	8
D	60	0/8	0	3	6
D 2x	125	0/7	0	1	5
Contr.	-	1/8	12	6	⩾10
E	25	1/8	13	5	6
E 2x	100	0/7	13	4	7
Contr.	-	1/8	20	7	⩾10
F	300	0/8	0	2	3
F 2x	350	0/8	0	0	1
Contr.	-	0/8	13	9	6

* Fever = 40°C

The experimental procedures have been reported previously (De Leeuw and Van Oirschot, 1985). Briefly, 2 groups of sero-negative pigs were vaccinated intramuscularly at 10 weeks of age, one of them was revaccinated 3 or 4 weeks later. A 3rd group served as non-vaccinated control. Intranasal challenge with a virulent AD virus strain was done 3 months after the 1st vaccination. Evaluation of vaccine efficacy was based on mortality, growth arrest, fever and virus shedding periods after challenge. A maximum of standardization was exercised.

Comparison of efficacy of an attenuated vaccine given intranasally with 2 inactivated vaccines given parenterally in pigs with maternally derived antibody.

In the Netherlands, over 90% of breeding pigs are vaccinated against AD, usually twice a year. In consequence, pigs possess MDA at the start of the fattening period. Table 2 shows that pigs born to sows vaccinated in the field during each pregnancy (4 to 6 weeks before parturition) with an inactivated vaccine (Nobivac® Intervet, the Netherlands) can have rather high serum-neutralizing (SN) antibody titres around 10 weeks of age. In addition, considerable variation exists in individual SN-titres.

TABLE 2 Maternal antibody titres in pigs born to sows vaccinated under field conditions.

Farm	Age (weeks)	No. pigs tested	Mean SN-titre	Variation SN-titre
A	8-10	70	54	12-384
B	10-12	19	35	12-128
C	10-12	35	42	24-128
D	10-12	24	18	6-48

The neutralisation test was done with an incubation period of the serum-virus mixture of 24 hours. Titres were expressed as the reciprocal of the final serum dilution inhibiting cpe in 50% of the cultures. An AD virus infection of these pigs could be excluded on the basis of supplementary serological investigations.

Maternal immunity does not protect the pigs during the entire fattening period. Thus, in regions where AD is enzootic, vaccination of fattening pigs with MDA may be necessary. However, maternal immunity interferes with production of active immunity after parenteral vaccination.

To overcome this, an IN vaccination with an attenuated virus has been developed (De Leeuw et al., 1982) and was subsequently compared with 2 inactivated vaccines as to efficacy. The vaccines used were the attenuated Bartha strain for IN vaccination (vaccine A), and the inactivated vaccines Geskyvac®, (Roger Bellon, France) (vaccine B), and Nobivac®, (Intervet, the Netherlands) (vaccine C). The results of 4 comparative experiments (Table 3) demonstrate that a single IN vaccination with the attenuated virus conferred a considerably better protection than parenteral vaccination with the 2 inactivated vaccines.

However, the efficacy of IN as well as parenteral vaccination decreased with increasing levels of MDA at vaccination. More detailed results on IN vaccination have been published elsewhere (De Leeuw and Van Oirschot, 1985; Van Oirschot and De Leeuw, 1985).

After IN vaccination the attenuated Bartha strain is excreted and this might result in transmission to susceptible pigs, and subsequently in serial passages over pigs. Passages over pigs may, theoretically, lead to reversion of virulence. We have made 6 passages with the non-plaque-purified Bartha strain in 2-day-old pigs and found no evidence for such a reversion. In another study, the Bartha strain could not be reactivated from pigs vaccinated intranasally at 10 weeks of age, suggesting that the strain does not induce latency (Van Oirschot and Gielkens, 1984). Thus, these often raised objections against the use of attenuated AD virus vaccines may not be justified, at least with respect to the Bartha strain.

TABLE 3 Summarized results of vaccination experiments in pigs with maternally derived antibody.

Exp. No.	Age at vaccination (weeks)	Vaccine	Mean SN titre		Challenge results			
			at vaccination	at challenge	No. dead	Mean days of Growth arrest	Fever	Virus shedding
1	10	A	4	40	0	3	3	5
		B	6	13	0	9	5	≥10
		Contr.	5	1	1	10	7	≥10
2	10	A	10	40	0	3	3	6
		C	13	100	0	5	5	ND
		Contr.	10	3	2	12	8	≥10
3	6	A	32	10	0	7	3	6
		C	40	20	0	11	5	9
		Contr.	40	3	2	11	5	≥10
4	9	A	50	20	0	6	4	8
	4+9	C, 2x	64	130	0	8	5	8
		Contr.	50	10	1	10	7	9

Pigs (7 or 8 per group) with various levels of MDA were vaccinated between 4 and 10 weeks of age and challenged 2 months after (the last) vaccination. The evaluation of vaccine efficacy was done as described previously (De Leeuw and Van Oirschot, 1985).

The above and other studies (McFerran et al., 1982; Zuffa et al., 1982; Pensaert and Maes, 1984) indicate that attenuated AD virus vaccines are generally more effective than inactivated vaccines. However, even the best attenuated vaccines cannot prevent the development of mild illness, the shedding of virulent virus and the establishment of latency. Most attenuated vaccines are derived from virulent AD virus by numerous serial passages in heterologous cells and as a consequence random mutations are introduced into the viral genome. In addition, most vaccine preparations are heterogeneous, that is they contain a population of closely related genetic variants (Gielkens et al., 1984) of which some may have different biological properties (unpublished data). The above observations indicate a need for more potent and, in molecular-virological respect, better defined AD virus vaccines.

Construction, virulence and immunogenicity of deletion mutants from virulent AD virus

As parent strain for the construction of a "live" bio-engineered AD virus vaccine a virulent and highly immunogenic virus (the Northern Ireland Aujeszky 3 (NIA-3) strain, kindly provided by Dr. J.B. McFerran) was used. This strain conferred complete protection in pigs, as evidenced by the results of an experiment

in which the immunogenicity of a thymidine kinase negative (TK$^-$) mutant from the NIA-3 strain was tested (Table 4).

TABLE 4 Protection induced in pigs by the NIA-3 strain and a TK$^-$ mutant of NIA-3

Virus	Mean SN titre at challenge	Challenge results				Rise in SN-titre after challenge
		Death	Growth arrest	Fever	Virus excretion	
NIA-3	630	0/3	0	0	0	-
NIA-3 TK$^-$	50	0/4	0	2	5	+
Controls	-	2/5	9	7	9	+

In this experiment a BUDR-induced TK$^-$ mutant of the NIA-3 strain was compared with a TK$^+$ plaque-purified virus of the NIA-3 strain with regard to efficacy. Pigs were inoculated intranasally with 10^5 PFU of virus and challenged intranasally with 10^5 PFU of the NIA-3 strain at 6 weeks after inoculation. Evaluation of protection was done according to our standard procedures.

After construction of hybrid viruses from the virulent NIA-3 strain and the avirulent NIA-4 strain and testing these for virulence in pigs it was concluded that genes mapping in the unique short (Us) and/or repeat region are involved in the expression of virulence (Berns et al., 1985). In a recent paper, Lomniczi et al. (1984) reported that Us genes are necessary for virulence of AD virus as determined in young chickens. We therefore constructed mutants with deletions in the Us region, starting from the NIA-3 strain.

The methods for the construction and characterization of deletion mutants will be described in detail elsewhere (Quint et al., submitted). Briefly, NIA-3 viral DNA was digested with endonucleases Bam Hl or Hind III. Bam Hl fragment 7 (Fig. 1) was cloned in plasmid pBR 322. This plasmid was linearized by partial Bal 1 digestion. Full-length DNA molecules were isolated, digested with exonuclease Bal 31 and recirculized. Two plasmids with deletions of 2.8 and 2.4 kbp in DNA fragment 7 were selected. From the molecularly cloned NIA-3 Hind III B fragment the MluI-Bgl II fragment was replaced by the MluI-Bgl II fragment of the 2 plasmids bearing the deleted inserts. The viral inserts of the final recombinant plasmids (pHB 2.8 and pHB 2.4) were mixed with the Hind III A fragment of NIA-3 virus, and transfected into secondary porcine kidney cells (Fig. 2).

Reconstituted viruses from transfections were plaque-purified 3 times and their genomes characterized by Southern Blot analysis, using digestions with Bam Hl, Sal 1, Bam Hl + KpnI, and Bam Hl + KpnI + Hind III, and probing with ^{32}P-labelled NIA-3 DNA or molecularly cloned subgenomic fragments.

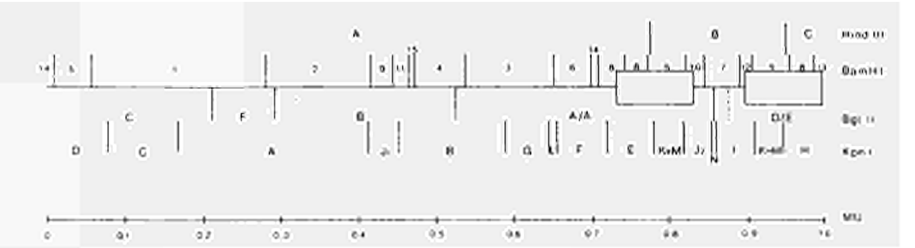

Fig. 1.
Physical map of the NIA-3 strain of AD virus. The sites for BamHI, Bgl II, Hind III and KpnI are presented. The rectangles represent the inverted repeats. For each enzyme, fragments are given in alphabetical or numerical order according to size. Identical sized fragments are indicated by a "prime".

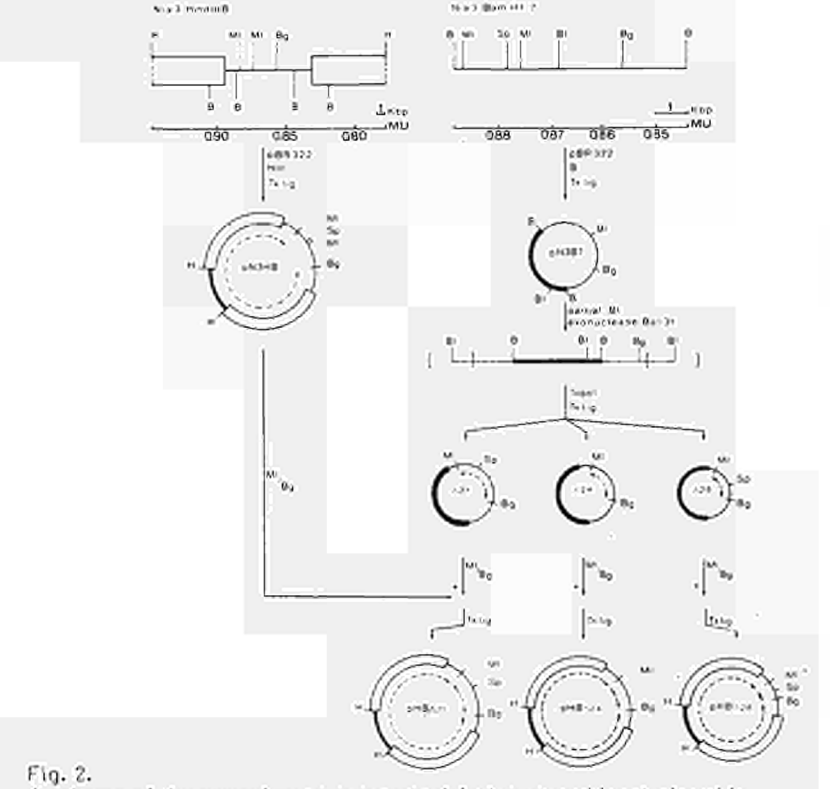

Fig. 2.
A scheme of the procedure to construct deletion recombinant plasmids

The deletion mutants were characterized as having a NIA-3 pattern with deletions of 2.8 kbp (2.8 N3A) and of 2.4 kbp (2.4 N3A) between map unit position 0.886 and the Bgl II site at position 0.856, involving the loss of the MluI site at position 0.876 (Fig. 1B). The mutant 2.4 N3A had lost the SphI site, whereas the mutant 2.8 N3A had retained it. In addition, both mutants had a small deletion (about 100 nucleotides) comprising the Hind III site in the inverted repeat region (Quint et al., submitted).

The mean time to death (MTD) in mice upon inoculation with AD virus strains has been used to group strains according to virulence (Platt et al., 1980). In general, virulent AD virus kills mice faster than attenuated strains, but at least one vaccine virus strain kills mice in the same time as a virulent strain (Van Oirschot and Gielkens, 1984). The 2 deletion mutants had significantly (Wilcoxon rank test, $p < 0,05$) longer MTD's than the parental NIA-3 strain (Table 5), indicating that a marker for virulence in mice is present in the Us and/or repeat region. The position and/or length of the deletion in the Us region may affect virulence, as shown by the significant (Wilcoxon rank test, $p < 0,05$) differences in MTD's of the 2 mutants (Table 5).

TABLE 5 Virulence tests of deletion mutants of AD virus

Virus	Mice	Pigs					
	MTD (hours)	Death	Growth arrest	Fever	Virus excretion		Mean SN-titre
					% pos	mean titre	
NIA-3	50 ± 4.0	2	9	4	90	3.6	700
2.4 N3A	73 ± 7.0	0	0	0	80	2.7	45
2.8 N3A	60 ± 8.5	0	0	3	50	2.2	42

Groups of 8, 6 to 7-week old Balb/c mice were inoculated subcutaneously with 10^6 PFU of virus in a 0.1 ml volume. The mice were monitored at intervals of 6 hours for 10 days. MTD (mean time to death) values are given with standard deviations.

Groups of 5 pigs were inoculated intranasally with 10^5 PFU of virus. Evaluation of virulence for pigs was done essentially as described previously (De Leeuw and Van Oirschot, 1985). % pos = percentage of positive oropharyngeal fluid samples collected the first 10 days after inoculation. Mean virus titre expressed as $\log_{10}$ PFU of virus per gram of fluid.

In the microneutralisation test a serum/virus incubation period of 24 hours at 37°C was used. Titres were expressed as the reciprocal of the final serum dilution inhibiting cytopathic effect in 50% of the cultures. Mean SN-titres at 3 weeks post inoculation are given.

Both mutants had a strongly reduced virulence for pigs. Whereas the parental NIA-3 strain killed 2 of 5 pigs, the deletion mutants induced no mortality. The pigs given the parental NIA-3 strain developed typical signs of AD, whereas the pigs given the mutant 2.4 N3A or 2.8 N3A only showed very mild depression or fever, respectively. The pigs inoculated with the mutants showed no growth arrest. The mutants were shed in oropharyngeal fluid to a lower level than the parental strain (Table 5). At 3 weeks post inoculation the pigs surviving the NIA-3 infection had more than 10 times higher SN-titres than pigs given the mutants. However, the latter pigs were well protected against a challenge with virulent AD virus 6 weeks post inoculation (Table 6). They developed no signs of disease, but did shed virulent virus, although at a much lower level than non-vaccinated controls. Particularly, mutant 2.4 N3A was able to considerably reduce virulent virus shedding and in 2 pigs given this mutant no virus shedding could be detected at all after challenge. This aspect is of high significance with respect to eliminaton of field-virus circulation.

The pigs given the mutants 2.4 N3A or 2.8 N3A appeared to be better protected than pigs vaccinated IN with 10^6 PFU (a 10 times higher dose than used in this study) of the Bartha strain (De Leeuw et al., 1982).

TABLE 6 Evaluation of protection induced by deletion mutants of AD virus

Virus	Mean SN-titre at challenge	Challenge results				
		Death	Growth arrest	Fever	Virus excretion	
					% pos*	mean titre
2.4 N3A	180	0	0	0	20*	0.5
2.8 N3A	110	0	0	0	52	2.0
Controls	-	0	20	8	100	5.3

Pigs given the deletion mutants and 2 sero-negative control pigs of the same age were inoculated intranasally with 10^5 PFU of the virulent NIA-3 strain, 6 weeks after inoculation. The challenge and evaluation of protection was done according to our standard procedures.
*Percentage of virus-positive oropharyngeal fluid samples during the first 10 days after challenge.

CONCLUSION

The results demonstrate that 2 genetically engineered deletion mutants derived from a virulent AD virus strain had a markedly reduced virulence, and were highly immunogenic in pigs. Using the present approach more modifications, if necessary, can be introduced into the genome of these mutants, for instance to affect the viral ability to establish latency, or to enhance immunogenicity.

Thus, the AD virus deletion mutants are considered to provide a good base for the production of bio-engineered "live" AD virus vaccines.

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SUMMARY OF SESSION SIX.

NOVEL APPROACH FOR THE PREVENTION OF HERPESVIRUS INFECTIONS.

by H. LUDWIG and L. THIRY.

Dr. BECKER reported on current developments in the field of vaccines. It was emphasized that a variety of live attenuated vaccines are known which induce a preventive immune response. As an example the yellow-fever virus vaccine was cited. Similar approaches have been made with herpesviruses. It is, however, neccessary to have more information on the genes coding for the immunogenic components, and more importantly, to know the genes responsible for virulence. Contributions of the STEVENS group and, in this meeting, of DARAI's group give promising insights into neurovirulence genes. The importance of the TK gene for a herpesvirus to go latent and/or pass the barrier to the brain was emphasized. TK^- viruses are known to reach the ganglion and cause latency. Furthermore, correlations of small and large plaque herpesviruses with virulence were presented.

A further approach includes recombinant DNA vaccines which are currently produced using parts of the vaccinia genome and the glycoprotein regions of HSV like the gB and the gD region in expression vectors. Promising observations appear to come from the yeast-system rather than the E. coli system.

A third attempt is chemical synthesis of immunogenic components (oligopeptides). This has successfully been done to synthesize gD products of HSV. These peptides are able to induce neutralizing antibodies and to protect mice. The problem with these experiments is that injection of virus and structural components does not reflect the natural way of a virus entering the organism. Dr. BECKER concluded that in current approaches to create save and efficacious herpesvirus vaccines it is of importance to present the immunogen in the right way for an effective activation of the immune response.

Dr. L. THIRY's question concerning the TK gene(s) in human cells could not be answered, since such genes are more precisely investigated in the mouse system. Dr. LUDWIG raises the question whether attenuation of herpesviruses through passages in cell cultures or susceptible animals might be a helpful way to select avirulent viruses. Like in the pseudorabies virus system deletions seem to occur always at the same genome location. Dr. GASKELL points to an observation with feline herpesviruses where small plaque variants always occur after reisolation of virus from infected cats. There is, however, no obvious rearrangement in the DNA of such viruses. Is the cat naturally selecting the small plaque virus? Dr. LUDWIG does not agree with the opinion that a plaque phenotype in general correlates with virulence. Dr. SUBAK-SHARPE mentions that many genes influence plaque form and plaque size and there is not enough information about the gene functions yet. It is of importance that attenuated virus keep on going to mutate, which makes it essential to continuously control whether the

immunogenicity of these viruses is not lost.

Dr. LUDWIG asks whether recombinations in nature might occur between wild type and attenuated or deleted viruses. Dr. SUBAK-SHARP mentions that recombination and reversion have to be kept apart. Reversion seldomly occurs. A recombination after superinfection with wild type virus is possible; the probability of its in vivo occurrence, however, is quite low. Dr. HILL asks whether one can regard latency as a good or a bad thing, since it is often established after immunization. Dr. BECKER states that it may be good in lymphocytes but deleterious in the nervous system. Dr. BECKER concludes with some suggestions on how to construct modern vaccines and emphasizes that a more rational approach (genetic engineering of vaccines) should be made, rather than producing vaccines on an empirical basis.

The following report by Dr. L. THIRY covers the efficacy and the problems arising with the introduction of a new varicella zoster virus life vaccine into human populations. Questions concerning the effectiveness of the VZV vaccine against chicken pox cannot be fully answered. The vaccine strain has a clearly different restriction enzyme pattern from that of the wild type strain. The alterations on the genome have not been mapped.

The last report by Dr. VAN OIRSCHOT deals with genetically engineered pseudorabies (PsR) virus vaccines. After it was detected that in vaccine strains, like the Bartha strain, deletions in the short unique region of PsV virus occur, Dr. VAN OIRSCHOT's group used this information to construct a new virus from two Northern Ireland strains. A virulent strain served for the part containing the unique long region and the non-virulent strain supplements the unique short region. The new virus has a characteristic deletion in the Bam HI fragment 7 similar to that of the Bartha strain. The biological properties of the virus can be summarized as follows: infected mice die after a long incubation period, the virus does not kill pigs and does not lead to growth delay. Dr. CASTRUCCI raises the question, whether this virus is safe for cattle and other animals, and mentions that any vaccine that concentrates only on one species cannot be valuable. Dr. BECKER points to the information that TK- mutants should be used for constructing these vaccines, as well. Dr. VAN OIRSCHOT refers to further experiments, which still have to be done.

In summary, this session presented information about two live-virus vaccines, one prepared from attenuated varicella-zoster virus, the other constructed from pseudorabies virus DNAs. These approaches show two extremes of current developments in vaccine production. First, creating vaccines on an empirical basis and secondly, engineering vaccines with, hopefully, less virulence. There is a general consensus that the control of herpesvirus infections by vaccination appears to be possible in some cases. In other cases we need more information on the viruses themselves and their interactions with their hosts.

SESSION 7

PROTECTION OF ANIMALS AGAINST HERPESVIRUS INFECTIONS

Chairman : O.C. STRAUB

Co-chairman : J.T. VAN OIRSCHOT

STUDIES ON EARLY PROTECTION AGAINST FELID HERPESVIRUS 1 (FHV-1) FOLLOWING INTRANASAL VACCINATION WITH A LIVE COLD ADAPTED FHV-1 STRAIN

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ABSTRACT

Intranasal vaccination with a cold-adapted strain of FHV-1 provides solid immunity against feline viral rhinotracheitis by 6 days post vaccination. The onset of protection at this time appears to be specific and has all the characteristics of a conventional immune response. However at 6 days post vaccination only low levels of FHV-1 specific IgA and IgM antibody can be found in serum and nasal secretions and no significant amounts of interferon are detectable. Also in lymphocyte transformation assays neither peripheral blood lymphocytes nor tonsil lymphocytes gave a significant proliferative response in the presence of FHV antigen.

Pathogenesis experiments demonstrated that the tonsil is an important primary site of virulent FHV-1 replication which was significantly reduced in the tonsils of vaccinated cats. This suggests that a local cell mediated immunity, possibly initiated within the tonsil, may play a more important role than local antibody in protection against FHV-1 infection.

INTRODUCTION

Feline viral rhinotracheitis virus, now known as Felid herpes-virus 1 (FHV-1) is a major upper respiratory pathogen of cats and was first isolated in 1957 by Crandell & Maurer (1958). It causes an acute febrile syndrome characterised by sneezing, depression, and copious ocular and nasal discharges. FHV-1 is also highly infectious and is a widespread problem in boarding catteries, breeding colonies and other situations where a large number of cats have been brought together. Infection is mainly by means of aerosols and the virus reproduces locally in the mucosae of the nasal turbinates and conjunctival regions (Gaskell & Wardley 1978); soft palate, tonsil and trachea may also be involved (Gaskell, 1975). FHV-1 is relatively fragile in the environment and relies on survival by sequential spread and

setting up a carrier state in its host. As a result of this persistence in the animal, vaccines have only been moderately successful in controlling FHV-1 infection.

Recently a live cold-adapted mucosal FVR vaccine (Duphar Veterinary) has been developed which is administered intranasally and so far evidence suggests that it prevents a carrier state when a cat is challenged with the field strain (Orr et al, 1980). This type of vaccination conforms to the natural route of infection and is thought to produce a localised response in the respiratory tract because protection is rapid and is reported to occur within 2 days post vaccination (Slater & York, 1976). However the protective mechanisms involved have not yet been established and have so far only been investigated in terms of serum antibody which remains undetectable until day 20 post vaccination (Orr et al, 1980).

In this paper we have made use of another distinct upper respiratory condition of cats caused by feline calicivirus (FCV). This is a RNA virus unrelated to FHV and can therefore be used to study the specificity of the protection provided by FHV vaccine. Cats were challenged with either FHV or FCV at 2 or 4 days post vaccination with the cold adapted FHV vaccine. Clinical disease and virus shedding patterns were then monitored to determine the time of onset and specificity of early protection.

Having established this pattern of development, the important sites of early resistance to field FHV replication in vaccinated cats were determined and compared with local and systemic antibody responses, IFN levels and FHV-specific lymphocyte transformation responses by peripheral blood and tonsillar lymphocytes.

MATERIALS AND METHODS

Experiment A

An experiment was designed to determine the specificity of protection afforded by intranasal vaccination using a cold-adapted FHV-strain (log_{10} 6.9 $TCID_{50}$ per cat, Duphar Veterinary). Sixteen cats of approximately 12 weeks old were placed in isolated groups of 4 (Gaskell & Povey 1979) and designated A, B, C and D respectively. Groups B and C were vaccinated and after 48 hours C was challenged intranasally with the virulent strain of FHV-1 (B 927, log_{10} 7.1 $TCID_{50}$/cat) and B with the unrelated virus FCV (69/1112 strain log_{10} 7 $TCID_{50}$/cat). Groups A and D served as challenge controls so that the pathogenicity of FHV and FCV

cells using Polyinosinic-polycytidylic acid (Richmond 1971) and its titre determined by the microplaque reduction technique using cowpox (Strain L97, Gaskell et al 1983) as an indicator virus.

The sensitivity of FCV and FHV to the IFN produced in vitro was then examined using the same microplaque reduction technique as above.

Blood samples for lymphocyte transformation assays were collected 6 days post vaccination in syringes containing 20 units/ml preservative free heparin. Lymphocyte transformation assays were also carried out on lymphocytes isolated from tonsils (Goddard et al 1984).

RESULTS

Experiment A

No clinical signs of disease were observed during the pretest period. No virus isolations were made from nasal specimens made at the time of grouping or on day 0. Vaccinating cats with FHV vaccine two days before challenge with virulent FCV provided no protection against clinical disease. The control group (A) had a total mean cumulative score per cat of 86 compared to 84 for the vaccinated group (B) and this difference was not significantly different (Wilcoxon's two sample rank test). Cats in both groups showed the same degree of disease which was typified by large inflamed mouth ulcers in all cases.

The FHV-vaccine did however provide partial protection against virulent FHV. Within 5 - 6 days after challenge vaccinated cats (Group C) showed mild depression, reduced appetite, transient pyrexia and some sneezing and slight ocular/nasal discharge with a total mean cumulative clinical score/cat of 26.9 ± 12.3. The unvaccinated cats (group D) showed marked pyrexia, reduced appetite, mild depression and marked ocular/nasal discharge. Sneezing, milk conjunctivitis and dyspnoea were also recorded. The total mean cumulative clinical score per cat for this group was 82 ± 18.6 which was significantly different than the vaccinated/challenged cats (Wilcoxon's 2 sample rank test $P < 0.01$).

However despite the reduction in symptoms virulent FHV was shed from vaccinated cats in group C and there was no significant reduction in shedding time compared to the control group D.

The above experiment was repeated using FHV without FCV and the virus shedding patterns quantified. Both nasal and oropharyngeal titres of virulent FHV from vaccinated/challenged cats peaked at 3 days post chal-

could be ascertained. The cats were examined daily for clinical signs of illness and the severity of the major signs assessed on a point scale from 0 (normal) to 4 (severe) and expressed as a clinical score (Gaskell and Povey, 1979). Nasal and swab specimens for virus isolation (Wardley et al 1974) were collected from all cats at the time they were first placed in isolation, on the day preceding day 0 and daily thereafter for 20 days. Where swabs contained both vaccine and virulent FHV-1 plaque size differences in feline embryo cells (FeA) were used to distinguish between them. Both viruses had been previously grown in FeA cells at 37°C with an overlay of methyl cellulose for 3 days and plaque diameters measured. This provided a standard size for each virus which could be compared with virus plaques from swab samples grown under similar conditions (Cocker et al 1984) Having established the specificity and degree of protection further challenge experiments were carried out using FHV only and cats were challenged at either 2 or 4 days post vaccination. One group was given the vaccine strain only. All cats were then monitored clinically and virologically for 20 days as described above. The nose and oropharynx were swabbed daily and virulent FHV-1 was quantified using a standard plaque assay system (Crandell et al, 1960).

Experiment B

A second study was conducted to determine the primary sites of early replication and thereby ascertain in which tissues resistance to infection was taking place in vaccinated cats. Four cats were housed in two isolated pairs. One group was vaccinated with the FHV vaccine and after 6 days all four animals were challenged with virulent FHV. Euthanasia was performed on the cats after a further 48 hours and the viral content in nasal turbinates, tonsil, soft palate, submandibular lymph nodes and conjunctiva analysed by tissue homogenisation (Gaskell & Povey, 1979).

Experiment C

A third study was undertaken to investigate the immune mechanism responsible for early protection following intranasal vaccination with the FHV vaccine. Sera and nasal washings were collected at day 0 and 6 days following vaccination. These were assayed for interferon (Campbell et al 1975) and for anti-FHV antibodies using a plaque reduction assay (Gaskell et al 1983) and also an immunoglobulin class-specific ELISA (Voller et al 1976). Cat interferon was produced in vitro from Crandell feline kidney

lenge (5 - 6 days post vaccination) then began to fall. (The peak titre recorded in nasal swabs was 10^4 pfu/ul compared to 3 x 10^2 pfu/ul in oropharyngeal swabs).

The virulent titres from challenged only cats however reached higher levels (mean 5 x 10^4 pfu/nasal swab sample) and fell only after 7 - 8 days post challenge.

The total amount of virus shed from the 4 vaccinated/challenged cats was 6.8 x 10^4 pfu/ul which was just significantly less than the total of 9 x 10^4 pfu/ul recorded from unvaccinated challenged only cats (Wilcoxon's two sample rank test, ($P < 0.05$).

The vaccine strain was isolated from both nasal and oropharyngeal swabs of the vaccinated only group for a period of 6 - 9 days and two out of four cats showed transient pyrexia ranging up to 40°C. and sneezing for 1 - 2 days only.

When cats were challenged 4 days after vaccination instead of 2 days no clinical disease was evident and virulent FHV was isolated at a very low level, 5 x 10^2 pfu per swab on one day from the nasal swab of one animal only. Four unvaccinated controls inoculated with virulent virus showed similar clinical signs and shed virus as before.

Experiment B

In unvaccinated cats challenged with virulent FHV significant levels of the virus were only found in the tonsils and nasal turbinates at 48 hours post challenge. The mean titre [illegible]rulent FHV in nasal turbinates was 10^4 pfu/mg wet tissue weight, and [illegible]onsil 7.5 x 10^2 pfu/mg wet tissue weight.

However in nasal turbinates and tonsils of cats that had been vaccinated 6 days prior to challenge with virulent FHV the levels of virus were significantly reduced. A mean of 15 pfu/ug wet tissue weight was recorded for nasal turbinates and 0.5 pfu/ug wet tissue weight for tonsillar tissue.

Experiment C

Interferon results

Using a microplaque reduction technique and cowpox as an indicator virus, only very low levels of interferon were recorded 6 days post vaccination. Out of 5 cats examined the highest titre recorded was 1:30 for nasal washings and 1:10 for serum.

Cat interferon was produced in vitro using polyinosinic-polycytidylic

acid. This antiviral activity was confirmed by demonstrating that its ability to cause a 50% reduction in cowpox plaques was relatively unaffected by ultracentrifugation and overnight dialysis at pH2, but severely reduced by trypsin treatment. Using a standard in vitro cat interferon preparation FHV and FCV showed a 50% plaque reduction with a 1:175 and a 1:100 IFN dilution respectively. At 6 days post vaccination, anti-FHV neutralising activity could not be detected in either serum or nasal washings by a plaque reduction assay. However, using the more sensitive ELISA system, low levels of IgM and IgA antibody were found in nasal washings. This low level antibody response was also evident in serum but was restricted to the IgM class.

Lymphocytes from blood and tonsils of vaccinated cats showed a good proliferative response to the non-specific T-cell mitogens concanavalin A and phytohaemagglutinin in an in vitro lymphocyte transformation assay. However no FHV-specific response was evident when these lymphocyte populations were cultured for 6 days with an inactivated preparation of FVR infected FEA cells. In contrast, blood lymphocytes from cats recently recovered from a natural FVR infection consistently responded to this same antigen preparation when cultured under similar conditions.

DISCUSSION

Vaccination of cats with a cold adapted FHV-1 strain four days before exposure to the virulent virus will protect against clinical disease. Since the incubation period of experimental FHV in this study was one to two days it is concluded that cats show resistance to infection by five to six days after vaccination (i.e. four days between vaccination and challenge plus the one to two day incubation period). The onset of protection therefore has all the characteristics of a conventional immune response and appears to be specific for FHV-1 because no protection was afforded against FCV. Only very low levels of interferon were detected in nasal and serum samples six days post vaccination. These levels are probably unimportant in early protection because FCV replication, which we have shown to be sensitive to interferon in vitro, was not affected by vaccination.

However, despite the evidence for a solid immunity providing specific protection by six days post vaccination, only very low levels of anti-FHV

neutralising activity were found in nasal washings and serum. Similarly blood and tonsil lymphocytes showed no proliferative response to FHV antigen. In the unvaccinated cat challenged with virulent FHV, the tonsil appears to be an important primary site of virus replication (Gaskell & Povey 1979). Therefore the very significant reduction in replication seen in vaccinated cats suggests that a local cell cytotoxicity response plays an important role in protection against FHV disease.

The importance of the tonsil in protection against disease has been demonstrated by several workers studying human tonsil cells. Recently Drucker et al (1979) have detected cell-mediated immune responses in human tonsils, and Ogra et al 1971 demonstrated that children undergoing tonsillectomy before vaccination respond less well to a primary local immunisation with live, attenuated polio than do control subjects.

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USE OF RECOMBINANT HUMAN INTERFERON (Hu-IFNα_2) IN BOVINE HERPESVIRUS 1 INFECTION IN CATTLE

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ABSTRACT

Bacterially produced human interferon (Hu-IFNα_2), which has a weak antiviral effect on the multiplication of Bovine Herpesvirus 1 (BHV 1) in vitro, was intramuscularly administered (10^6 IU/kg body weight) daily during 6 days to young heifers, beginning one day prior to intranasal infection with 5 10^7 PFU of BHV 1. Treatment with Hu-IFNα_2 did not reduce the virus excretion nor the nasal discharge. Moreover, establishment of BHV 1 latency was not prevented, as demonstrated by viral reactivation following dexamethasone treatment. However, the injected interferon was biologically active. Indeed, in the treated animals, IFN induced the 2'-5' oligoadenylate synthetase activity and blocked the production of endogenous interferon.

INTRODUCTION

Bacterially produced human interferon Hu-IFNα_2 (Nagata et al., 1980) was shown to be clearly active in vivo against experimental vaccinia virus infection in young calves (Goossens et al., 1982 ; Wérenne et al., 1983, 1985). A daily treatment schedule at a dose of 10^6 IU/kg body weight provided complete protection in some animals. Although this experimental system was particularly convenient to quantify the protection conferred by interferon treatment, it remained to be determined if human interferon might be useful for the treatment of some important viral diseases of cattle. We have already shown that Rotavirus induced newborn calf diarrhoea is totally inhibited by exogenous Hu-IFNα_2 (Schwers et al., 1985).

The fully successful protection against this important viral disease we have obtained confirmed the role of endogenous interferon in the control of pathogenicity of Rotavirus we had demonstrated before (Vanden Broecke et al., 1984). We had shown indeed that in conditions where endogenous interferon is produced very early after infection with Rotavirus (using a very high dose of virus) no sign of pathogenicity was observed. On the contrary, in conditions where an identical level of interferon is produced but much later on (using a small dose of virus), pathogenic symptoms fully developed. The time of establishment of the antiviral state is therefore more essential than the level of circulating interferon produced. A similar situation could therefore be expected by analogy in other viral infections.

Infectious bovine rhinotracheitis is economically among the most important infections in cattle. In fact, the pathology of infection is largely due to respiratory infection. Other symptoms can also be observed : infectious pustular vulvovaginitis, enteritis, conjunctivitis, abortion, encephalitis in young calves. Newborn calves like foetuses suffer from an acute and systemic fatal infection, but present also, sometimes, the respiratory disease (Pastoret and Thiry, 1984).

With BHV 1, known to be a good interferon inducer (Ahl and Straub, 1971 ; Rosenquist and Loan, 1969), a temporal relationship between interferon in respiratory tract secretions after avirulent IBR treatment and onset of protection against virulent BHV 1 challenge was described in some studies (Todd et al., 1972) but not in other (Savan et al., 1979). As we showed that BHV 1 is inhibited in vitro by recombinant Hu-IFNα_2 (Goossens et al, 1983a) even if it is less susceptible than was VSV or Rotavirus (Goossens et al., 1983b), we decided to undertake an in vivo study also. We tested the antiviral effect of bacterially produced human interferonα_2 on the primary infection of cattle by BHV 1 as well as the effect on the treatment on the establishment of BHV 1 latency. For this purpose, we used Hu-IFNα_2 Arg supplied by Boehringer Ingelheim.

MATERIAL

Interferon : Lyophilized bacterially produced human interferon Hu-IFNα_2 Arg (specific activity of 3 10^5 IU/mg protein) was supplied by Boehringer Ingelheim Vetmedica. The interferon preparation contains human serum albumin (15 mg HSA/ 5 10^6 IU). The in vitro activity of IFN

preparation was tested against the LA strain of BHV 1 by a plaque reduction assay in GBK cells.

Virus : The strain IBR/Cu 5 of Bovine Herpesvirus 1 (BHV 1) which was isolated in Belgium from a respiratory case (Pastoret et al., 1979) was used for cattle infection. The strain IBR/LA was used for seroneutralization procedure.

Experimental animals : Eight Holstein friesan heifers 6-8 months of age and devoid of serum neutralizing antibodies against BHV 1 virus were obtained from a single source.

METHODS

Titration of nasal virus excretion : Ten fold dilution of virus suspensions were adsorbed to monolayers of Georgia Bovine Kidney (GBK) cells grown in microplates. Plaques were obtained under MEM supplemented with 5% specific bovine anti BHV 1 serum (neutralization antibody reciprocal titre : 64). After three days of incubation, cells were fixed and stained with an hydro-alcoholic solution of crystal violet. Plaques were counted.

Interferon titration : Circulating antiviral activity was determined in the plasma by the classical technique of reduction of cytopathic effect (Stewart, 1979) in GBK cells, using as challenge Vesicular Stomatitis Virus (VSV).

Determination of the lymphocytic 2'-5' oligoadenylate synthetase activity : Lymphocytes were isolated from blood by Ficoll-Hypaque density centrifugation, divided into aliquots (4 10^6 cells/tubes) and stored at -70°C. Cell extracts were prepared by Nonidet P-40 lysis (Williams et al, 1981) and 2'-5'A synthetase activity was measured in solution assays containing 10 µl of extract, 20 mM Hepes buffer pH 7.5, 25 mM $Mg(OAc)_2$, 5 mM ATP, 1 mM DTT, 4 mM $Fru\text{-}P_2$, 20 µg/ml Poly IC, 0.5 µl (^3H) ATP (NEN, 47 Ci/mmol) in a final volume of 25 µl. The 2'-5' oligoA synthesized were isolated by DEAE-cellulose chromatography as described by Minks et al. (1979).

Determination of antibodies against BHV 1 : Two-fold serum dilution were reacted with 100 plaque forming unit (PFU) of BHV 1 and tested by a micromethod using GBK cells maintained in MEM supplemented with 5% foetal calf serum (FCS). Titres represented the reciprocal of the highest serum dilution which inhibited 50% of cytopathic effect.

Experimental schedule and procedures :

1. Grouping of animals : 8 BHV 1 seronegative animals were divided into three groups. Group I. Three heifers (305, 460, 785), maintained outside, were infected with BHV 1.

Group II. Three heifers (307, 461, 781), maintained outside and separated from the 3 first animals were infected with BHV 1 and treated with Hu-IFNα_2.

Group III. Two heifers were kept together in a stable : heifer 457 was considered as a " negative control " , heifer 409 only received Hu-IFNα_2 (IFN control).

2. Primary infection with BHV 1 : On day 0, animals of the two first groups (I, II) were infected with 5.10^7 PFU of IBR/Cu5 strain of BHV 1. Nasal swabs were taken daily during 15 days. Swabs were weighed before and after sampling. After sampling, they were eluted in 2 ml MEM. Virus titration was performed as soon as possible and virus excretion was expressed in PFU/ 100 mg of nasal exsudate. Serum samples were regularly (7 days interval) collected for neutralization and were heat-inactivated. Hu-IFNα_2 (10^6 IU/kg) was intramuscularly injected daily, from day - 1 to day 4 (6 injections) to the animals of group II and one animal of group III (409). The other animal of group III (457) as well as the animals of group I were intramuscularly (i.m.) with physiological serum, following the same schedule. To measure the circulating antiviral activity and the level of 2'-5'A synthetase activity in lymphocytes, blood samples (20 ml) were collected in heparinized tubes at different times : at t=0h, 1h, 8h on day -1 and at t=0h on days 0,1,2,3,4,5,6,8,10,16 (t=0h corresponding to the time of IFN injection). Body temperature was taken daily.

3. BHV 1 reactivation with dexamethasone treatment: Reactivation assay started after three months (day 84) by giving to all the animals five consecutive daily intravenous injections of dexamethasone (Fortecortine Bayer : 0.1 mg/ kg body weight). Body temperature was taken daily. Nasal swabs were collected daily during 15 days and treated as previously described. Serum was taken regularly for neutralization. Blood samples were collected (20 ml) daily until day 11 days after the beginning of reactivation to measure the circulating antiviral activity and the lymphocytic 2'-5'A synthetase activity.

RESULTS

1. Effect of Hu-IFNα_2 Arg on BHV 1 replication in vitro

BHV 1 replication in GBK cells is weakly sensitive to the antiviral effect of Hu-IFNα_2 Arg. Indeed, even with 10^3 IU/ml of interferon, only a reduction of 50% of BHV 1 plaque formation can be observed (Figure 1).

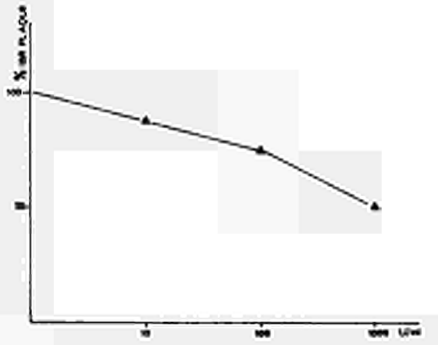

Fig. 1. In vitro effect of Hu-IFNα_2 on the formation of BHV 1 plaques in bovine GBK cells.

2. Primary BHV 1 infection

All the animals infected with BHV 1, treated or not with interferon excreted BHV 1 virus from day 1 to day 9, day 0 being the day of virus inoculation. The maximum of nasal virus excretion was localized between days 2 and 4. Ten days after the virus inoculation, none of the animals excreted virus anymore (Figure 2).

Although the disease symptoms seemed somewhat less important in the treated animals (nasal discharge, red nose ...), replication of BHV 1 was not inhibited in the nasal tractus after intramuscular injection of Hu-IFNα_2 Arg. All the 6 animals inoculated with BHV 1 had fever. Temperature increase measured every 24 hours (before IFN injection in the treated animals) reached a maximum of 1.2°C on day 4. This increase corresponded to the development of the disease ; indeed, on the same day, the symptoms were clearly observable.

After a single injection in the control animal, circulating antiviral activity was already detectable 60 minutes after injection and was complete-

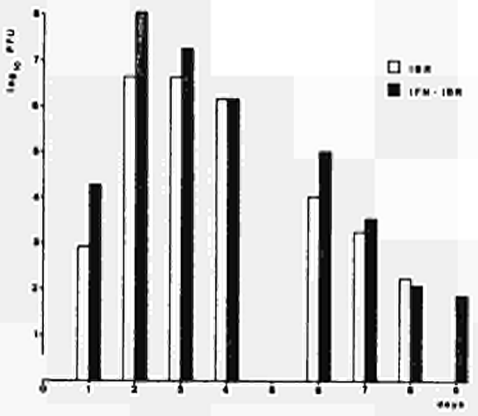

Fig. 2. Profile of nasal virus excretion during the primary infection with BHV 1, starting on day 0

ly cleared out from the circulation after 24 hours. Hyperthermia, which is now described as a classical effect of interferon, was maximum (+ 1.5°C) after 8 hours and disappeared after 24 hours. A two fold increase of the lymphocytic 2'-5'A synthetase level could be observed already after this single injection (data not shown).

Kinetics of circulating antiviral activity apparition was studied on day - 1 in the three treated animals (BHV 1/ IFN). It was similar to what has been observed in heifer 409 (IFN control) : the maximal IFN level obtained after one hour was 10^3 IU/ml. No more interferon could be detected 24 hours after the injection. No IFN accumulation was detectable at the end of the daily treatment.(Figure 3). In the plasma of the 3 infected heifers, not treated with interferon,a peak of antiviral activity could be detected by the classical test of reduction of VSV cytopathic effect in bovine GBK cells (Figure 4). This peak localized between day 2 and 4 indicates that relatively high level of circulating interferon (200 to 10^3 U/ml) resulted from viral infection. This peak was not present in the plasma of IFN/ BHV 1 heifers.

Modifications of the lymphocytic 2'-5'A synthetase activity :

During this Hu-IFNα_2 Arg repeated treatment (a total of 6 i.m. injections) the maximal level of 2'-5'A synthetase activity in the lymphocytes (4

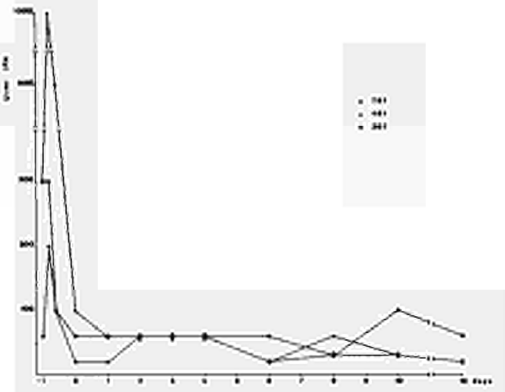

Fig. 3. Antiviral activity detected in the plasma of the animals of group II, infected with EMV 1 and treated i.m. with Hu-IFNα_2 Arg (10^6IU/kg body weight).

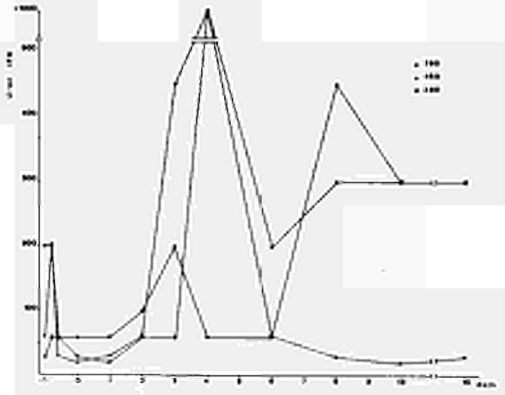

Fig. 4. Antiviral activity detected in the plasma of the animals of group I, during the primary infection with EMV 1.

fold increase) was reached after two injections (Figure 5). This level was maintained until the end of the treatment (day 4). Twelve days after the last injection (day 16), the lymphocytic level of 2'-5'A synthetase was identical to the basal level exisiting before the beginning of the experiment. The increase in activity of 2'-5'A synthetase was related to the level of circulating interferon ; the maximal increase of the 2'-5'A synthetase activity was observed in the lymphocytes of the heifer 461 which had the highest plasmatic interferon level one hour after injection (data not shown). A single IFN injection was not sufficient to reach the maximal level of the enzyme activity : as mentionned above, a two fold increase in the 2'-5'A synthetase activity was obtained in this case.

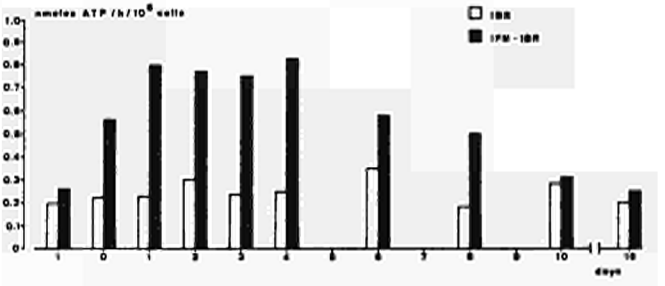

Fig.5. Induction in the 2'-5'A synthetase actvity in lymhocytes of the heifers infected with BHV 1 treated or not with Hu-IFNα_2 (Mean of the results obtained on 3 animals in each group).

Without interferon treatment, in the three infected animals, the 2'-5'A synthetase activity remained constantly low during the first 4 days of BHV 1 infection. The enzyme level was however slightly increased on day 6, two days after the maximal level of endogenous antiviral activity was reached (see the mean values in figure 5).

Production of anti BHV 1 antibodies : Production of anti BHV 1 antibodies occured already two weeks after BHV 1 inoculation : it was identical in the two groups of animals. This level remained constant until the beginning of the dexamethasone treatment.

3. BHV 1 nasal reexcretion by dexamethasone treatment.

Daily injections (for 5 days) of dexamethasone to the heifers, previously infected with BHV 1, reactivated BHV 1 nasal secretions. This reexcretion was maximal from day 4 till day 8 after the first dexamethasone injection and stopped 14 days after the beginning of the treatment (Figure 6). Again , no difference in the clinical symptoms was observed between the two groups of animals. After dexamethasone treatment, no endogenous IFN production could be detected by the CPE test on GBK cells neither in the plasma of heifer 409 (IFN control) nor of the 6 heifers reexcreting BHV 1 virus particles. A very slight increase of the 2'-5'A synthetase activity was however observed in the lymphocytes of the animals from both groups (Figure 7).

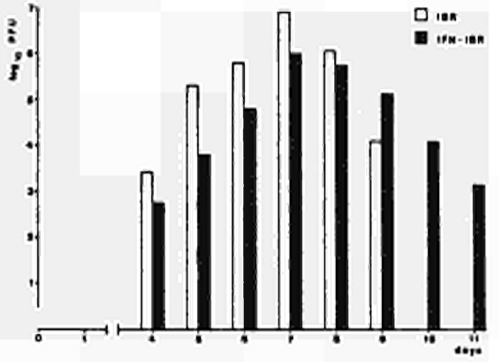

Fig.6. Profile of BHV 1 reexcretion following dexamethasone treatment (5 daily consecutive i.v. injections of dexamethasone at a dose of 0.1 mg/kg).

Nine days after the beginning of virus reexcretion, production of anti-BHV_1 bodies was enhanced in all animals : the titre obtained varied at the time between 32 and 128 (Table 1).

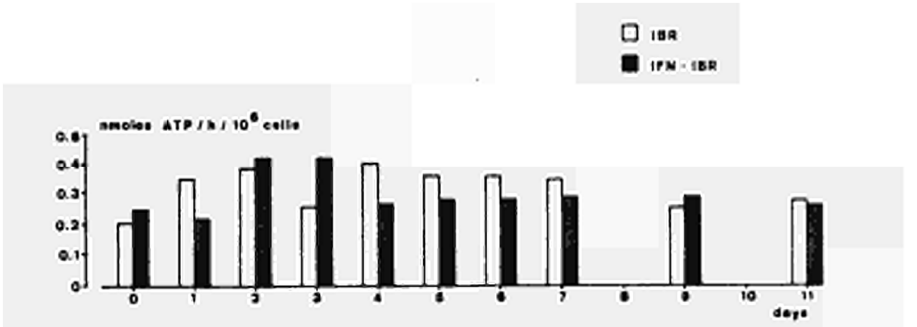

Fig.7. Level of the 2'-5'A synthetase activity in lymphocytes after dexamethasone treatment.

	DAYS	0	8	16	23	30	43	58	73	83	90	97
GROUP I	305	0	0	6	8	8	4	4	4	0	8	128
	460	0	0	4	8	8	4	8	4	0	4	64
	785	0	2	16	4	8	8	8	8	8	4	64
GROUP II	307	0	2	16	8	8	16	8	16	8	8	32
	461	0	0	4	16	8	8	8	8	16	8	64
	781	0	2	4	8	8	4	4	8	8	8	64
GROUP III	409	0	0	0	0	0	0	0	0	0	0	0
	467	0	0	0	0	0	0	0	0	0	0	0

Table 1. Evolution of the anti BHV1 antibodies level during all the experimentation (day 0 : primary infection ; day 83 : beginning of the dexamethasone treatment.

DISCUSSION

Intramuscular injection (i.m.) of bacterially produced human interferon (Hu-IFNα_2 Arg) in the bovine species did not suppress viral replication in heifers intranasally infected with BHV 1. On the basis of the data obtained with the small group of animals studied, we may not conclude for a statistically significant reduction in any particular symptoms

(temperature, nasal discharge) resulting from the viral infection, after IFN treatment. Further study using a lower viral infectious dose, closer to what occurs generally in natural infection, would be necessary to clarify the situation. Moreover, the establishment of viral latency (as revealed by virus reactivation with dexamethasone treatment) was not suppressed either. Viral multiplication in the nasal tractus being not affected by interferon, we would conclude, if the site of latency establishment is the same as the site of primary infection, that IFN has no specific effect on the critical step involved in the process. However, it is not excluded that latency results from viral infection at a secondary site. Lymphocytes could be good candidates. Again we could infer that such process is not affected by interferon treatment in the present conditions.
On the other hand, biological activity of Hu-IFNα_2 Arg in cattle was nevertheless clearly demonstrated in the present study :

- 2'-5'A synthetase activity was induced in lymphocytes of the animals treated with interferon
- no endogenous antiviral activity was induced, in contrary to what was observed in the infected untreated animals.

The biological events responsible for the induction of this endogenous activity were therefore inhibited by interferon.
Whether the absence of effect of interferon on the disease is due to the fact that, as we have shown in vitro, BHV 1 is relatively weakly susceptible to interferon or to the fact that the type of interferon administration used did not result in an appropriate biodisponibility of IFN can not be deducted on the basis of our present data. If it is the first possibility,we may have more chances to observe a protective antiviral effect with a lower viral infectious dose. If the second possibility holds, a local treatment with interferon, using an airspray system might give perhaps better results. Therefore before excluding the usefulness of interferon treatment for protecting cattle against infectious bovine rhinotracheitis , further studies are necessary.
Even, combined therapeutics, using other antiviral agents, including other interferon subtypes known to have other antiviral patterns, might be worthwhile to test, if appropriate conditions for a therapeutic action with Hu-IFNα_2 Arg can not be established.
The total success we have obtained (Schwers et al., 1985) in protecting newborn calves against rotavirus induced diarrhoea encourages us to pursue

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into this direction. Moreover, interesting data could be generated by such studies. The observations we have already made, showing some biological effects of interferon in BHV 1 infected heifers, indicate that interferon may be a fascinating tool to clarify some aspects of pathogenesis due to this virus, including latency.

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SUMMARY OF SESSION SEVEN.

PROTECTION OF ANIMALS AGAINST HERPESVIRUS INFECTIONS.

by O.C. STRAUB and J.T. VAN OIRSCHOT.

The two presentations of this session dealt with protection against herpesvirus infections. COCKER (United Kingdom) studied the mechanisms underlying the early protection after intranasal vaccination of cats with a cold adapted felid herpesvirus 1 strain.

To determine whether non-specific immune mechanisms were involved cats were infected with felid calicivirus. Four to six days after intranasal vaccination cats are well protected against challenge. At six days after vaccination low or no levels of interferon, neutralizing antibody and specific IgM, IgG and IgA were detected in serum and nasal washings. Peripheral blood lymphocytes and tonsillar lymphocytes were poorly stimulated by antigen at that time. Virus replication is markedly reduced in the tonsils of vaccinated cats. It is therefore suggested that local cell-mediated immune mechanisms are more important than local humoral immune responses in protecting cats against felid herpesvirus 1 infection.

Although intramuscularly administered, bacterially produced human interferon α2 was biologically active in young heifers, it was not able to reduce the severity of clinical signs, or to curtail viral excretion after intranasal infection of young heifers with bovine herpesvirus 1. In addition, this product was not able to prevent the establishment of bovine herpesvirus 1 latency (VANDEN BROECKE, Belgium).

SESSION 8

CLOSING REMARKS

IMMUNITY TO HERPESVIRUS INFECTIONS IN DOMESTIC ANIMALS
SUMMARY AND CONCLUSIONS

Y. Becker
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Jerusalem, Israel

The subject of this seminar has been the immune response of domestic animals to infections by herpesviruses. However, the discussions focussed mainly on new approaches for the development of live virus vaccines based on genetic engineering technologies. Live virus vaccines, such as herpesvirus of turkeys (HVT) or attenuated Marek's disease virus (MDV), that are used to immunize chickens against Marek's disease and pseudorabies virus used in pigs against Aujeszky's disease are derived from naturally occuring virus isolates. It became clear, as the seminar progressed, that it is essential to understand how the animal responds immunologically to infection by a pathogenic wild type virus, and how infection with a live attenuated virus leads to protection and immunity. I will try to summarize the major points raised in the contributions detailed in this book as an integrated subject, without citing all the individual contributors.

Viruses dealt with in the seminar

The viruses dealt with are listed in Scheme 1. Most are herpesviruses causing diseases in domestic animals (bovine, goat, pig, cat) and birds (chicken, pigeon). Studies on the human herpes simplex virus were presented as a molecular model, since existing knowledge on this virus is far more advanced than on any herpesvirus of animals. Varicella-zoster virus was discussed as an example of the use of an attenuated, non-engineered, vaccine for immune-depressed cancer patients.

Virus vaccines

The different types of virus vaccines which were discussed in the seminar are presented in Scheme 2. Live attenuated pseudorabies viruses are currently used to immunize pigs against Aujeszky's disease. Studies on the Bartha strain of Aujeszky's disease virus revealed that a mutation in the viral thymidine kinase (TK) gene leads to lack of TK expression (TK^- mutants) and to complete attenuation of the virulence of the virus. This is

Scheme 1 Viruses discussed in seminar

A. Bovine herpesvirus 1
   Bovine herpesvirus 2
   Bovine herpesvirus 4
   Goat herpesvirus
   Aujeszky's disease virus
   Feline herpesvirus
   Pigeon herpesvirus
   Marek's disease virus

B. Herpes simplex virus
   Varicella-zoster virus

Scheme 2 Virus vaccines

A. Attenuated virus
B. Inactivated virus
C. Subunit virus vaccine
   Viral glycoproteins made in
      E. coli
      Yeast
      Mammalian cells

D. Recombinant poxvirus
   Transfer of GP genes from
   herpesvirus to a poxvirus

similar to herpes simplex virus type 1 (HSV-1) in which a TK^- mutation, or deletion of the TK gene, causes attenuation of the virulence for mice. It was indicated that HSV-1 TK^- mutants can replicate in the nervous system of young mice (during the first 20 days after birth), when the nervous system thymidine kinase gene is still expressed and the immune system is not yet fully developed. Genetic engineering technologies make it possible to delete the TK gene from HSV-1, and thus to construct attenuated virus strains.

In addition to the HSV-1 TK gene which is involved in pathogenicity and allows the virus to invade the ganglia and brain stem of infected mice, an additional neurovirulence gene was discovered. This gene controls the ability of HSV-1 to replicate in the central nervous system (CNS). It was demonstrated that a deletion in the DNA in a naturally occurring HFEM strain of HSV-1 markedly reduced the pathogenicity of the virus. Recombination between HFEM DNA and a DNA fragment isolated from a pathogenic (F) strain of HSV-1 during transfection of cells in-vitro, led to transfer of the gene for virulence to the avirulent strain HFEM (Rosen and Darai, this volume; R.L. Thompson, E.K. Wagner and J.G. Stevens, Virology 131: 180, 1983).

Modifications of the DNA genome of Aujeszky's disease virus, using genetic engineering techniques, will, it is hoped, lead to the development of an engineered live vaccine strain for vaccination of pigs (Van Dirschot, this volume).

The use of inactivated herpesvirus vaccines for the immunization of domestic animals was also discussed. It was felt that live attenuated vaccine strains will be safe to use in domestic animals only when other animal species living around the vaccinated animals will not be endangered. Attenuated live virus vaccines can induce an immune response at the same site of entry as the wild-type virulent virus in the infected animal. In this context, the possible use of viral glycoproteins derived from cloned viral genes which can be expressed in E. coli, yeast, or mammalian cells, was discussed. All these procedures are based on the cloning of the relevant herpesvirus glycoprotein genes in a variety of expression vectors (plasmids). The viral glycoproteins can be isolated by passing cellular homogenates through columns with the specific monoclonal antibodies. The usefulness of these viral antigens for immunization of domestic animals will ultimately be dependent on the cost and availability of recombinant live attenuated herpesvirus vaccines.

Another possibility for the development of a recombinant virus vaccine was briefly discussed, namely the insertion of herpesvirus glycoprotein genes into poxviruses,as reported by B. Moss and collaborators (M. Mackett, G.L. Smith, B. Moss., J. Virol. 49:857, 1984). The use of such a recombinant vaccinia virus also depends on the removal of pathogenic viral genes from the poxvirus DNA genome.

Some of the problems arising from the immunization of domestic animals with a live virus vaccine were discussed. It will be necessary to consider the age of the animals to be immunized, the site of virus inoculation, the ability of the live herpesvirus to become latent, the ability of the immunized animal to shed the virus, and the ability of the virus to infect other animal species on the farm.

Infection with herpesviruses and the immune response

Scheme 3 depicts the sites of herpesvirus infection in domestic animals and in experimental animals discussed in the seminar. The initial step of the infection process (as summarized in Scheme 4-1), is the adsorption of the herpesvirus to cells at the port of entry, most probably the epithelial cells (keratinocytes). The skin-associated macrophages, Langerhans cells,

Scheme 3 Site of virus infection

A. Natural	B. Experimental
Mouth	Intradermal
Nose	Intravenous
Eye	Intracerebral
Vagina	

may also be involved. Replication of the virus in the infected cells leads to the release of the virus progeny to further infect neighboring cells. The viral glycoproteins are inserted into the outer membranes of the infected cells; the gE of HSV-1, for example, serves as an Fc receptor on the surface of the infected cell (M.F. Para, R.B. Baucke, P.G. Spear, J. Virol. 34:512, 1980). At this stage of infection, the herpesvirus can be isolated for diagnosis of the disease-causing virus.

The immune system responds to the virus infection (Scheme 4-2): viral antigens in the lesion are presented to subsets of T and B lymphocytes, antibodies are produced and released locally into the blood. At this stage, the disease can be diagnosed serologically. Macrophages enter the lesion, and eventually a healing process will start.

After the destruction of infected cells in the epidermis, the virus finds nerve endings and becomes attached to them (Scheme 4-3). Entry into the neurites signals the escape of the virus from the inhibitory effects of the immune system. With the axonic flow, the virus reaches the nucleus of the nerve cell in the corresponding ganglion. In the nucleus, the virus replicates, infects other ganglia and supporting nerve cells, and eventually continues its way through the axons to the brain stem and the CNS. A TK^- mutant of HSV-1 is unable to infect the ganglion. Under conditions which prevent the herpesvirus from entering the CNS, the virus remains latent in the ganglion and can be reactivated at this site (Scheme 4-4). Reactivated virus returns through the axons to the skin and replicates in epithelial cells, causing a recurrent lesion. It is assumed that interferon made locally in the ganglion leads to inhibition of virus infection and to virus latency. Processes leading to virus reactivation in neurons of the ganglia were discussed (Hill, this volume).

At the site of the virus lesion, herpesviruses might have the ability to infect lymphocytes (Scheme 4-5), in which the virus can remain dormant until the cells are stimulated to synthesize DNA. At this stage, virus replication is reactivated, and virus infection is possible. The immune

Scheme 4 Cellular and immunological responses to herpesvirus infection in animals

1. Infection
   - Adsorption of virions to cells
     - Epithelial cells
     - Langerhans cells (Fc receptors)
   - Replication of virus
     - Expression of viral genes
     - Viral glycoproteins on cell membranes:
     - HSV-1 gE is the Fc receptor
     - gD on cell surface
   - Diagnosis: Virus isolation

2. Immune system responds
   - Viral antigens are presented to T and B lymphocytes
   - Antibodies are made
   - Macrophages enter lesion
   - Diagnosis: Serological tests; neutralization test
   - Infected cells shed virus

3. Virus attaches to nerve endings and enters neurons
   - Infection of neurons in ganglia
   - Interferon produced locally
   - Virus latency in neurons

4. Reactivation of virus
   - Lesion on epithelium
   - Virus shedding
   - Rise in virus antibody titer
   - Spread of virus

5. Lesion develops further
   - Virus particles in fluid
   - Lymphocytes adsorb virus
   - Virus latency in lymphocytes
   - Virus replicates when lymphocytes are stimulated to divide

6. Immune response necessary to limit the lesion
   - Failing immune system leads to further increase in size of lesion
   - Active immune response leads to healing processes
   - Scar formation

7. Generalized disease
   - Virus infects RES cells (Kupffer cells in liver, etc.)
   - Virus enters spinal cord
   - Virus enters brain
   - Encephalitis

responses are needed to limit the development of the lesion at the site of virus infection (Scheme 4-6). A depressed immune system fails to limit the development of the lesion, whereas an active immune response leads to healing of the lesion. In immune-deficient patients, the herpesvirus can cause a generalized disease, most probably by infecting reticuloendothelial cells (e.g. Kupffer cells in the liver, etc.; Scheme 4-7). Such an infection can lead to the entry of the virus into the spinal cord, and eventually into the CNS, leading to brain damage and death.

The events described in Scheme 4 apply to all the animal herpesviruses discussed in the seminar (Scheme 1). It is hoped that the impact of the

seminar will be felt as work continues on the immune responses to herpesviruses, and new data will be provided on the mechanisms of infection and immunity in domestic animals.

Conclusions

The practical conclusions have been summarized by Dr. P.P. Pastoret, to whom we are indebted for organizing this seminar (together with Dr. E. Thiry). The need for live attenuated vaccine strains for effective and inexpensive immunization of domestic animals became obvious during the seminar. It seems that the genetic engineering approaches for the development of such vaccines were clearly pointed out. Many aspects of the sequence of events in infection and the responses of the immune system to herpesviruses are still unknown and require further investigation.

It was felt that the seminar has not only added further information on the role of the immune response in animals in the defense against herpesvirus infections, but has also added to our understanding of the nature of new biotechnological possibilities for future research.

RECOMMENDATIONS FOR FURTHER RESEARCH.

P.-P. PASTORET, J. SALIKI and E. THIRY.

As was pointed out during the opening session of the meeting, latent herpesvirus infections are of great importance for domestic animal health and production and the phenomena of latency, reactivation and re-excretion impede their control, because of their crucial epidemiological significance. Therefore studies on immunity to herpesvirus infections of domestic animals should lay more emphasis not only on the mechanisms that can play against the deleterious effects of a primary or secondary infection but also on those that can control viral re-excretion and, consequently, viral dissemination. The same is true of the next generation of vaccines. Their conception should take into account both the prevention of the effects of an infection and the control of viral excretion and dissemination by the animal. For this purpose, more basic knowledge is needed to upgrade our understanding of the precise role played by the various immunogenic components of the different herpesviruses that infect domestic animals, making use of what is already known on human herpesviruses.

The new data presented during this meeting were in favour of attenuated live vaccines which offer to the animal the most complete range of immunogenic components necessary for a well-balanced immune response. The attenuated live vaccines now available were produced rather empirically. Prospects of producing such vaccines by genetic engineering techniques appear nowadays to be more rewarding than the now classical approach of producing sub-unit vaccines. New horizons are now open for the construction of attenuated strains at will; attempts should be made to localize and delete "unwanted" viral genes, such as virulence genes, without altering the panoply of necessary genes. Efforts should also be directed towards the localization and eventual deletion of latency genes, in order to produce strains devoid of the side-effect of latency, if it is considered to be an undesirable effect. The attenuated strains now available remain in the organism in a latent state and the virus can be disseminated later on, virtually indiscriminately.

Since the pattern of the immune response, and especially of the immune control of re-excretion, changes with time, further research work should also be done on vaccination procedures. Furthermore, the consequences of latency are so complex that we need a new conceptual approach that may help to better our understanding of the interactions between immune mechanisms and the herpesviruses; such an approach may also be useful in evaluating *a priori* the impact of vaccination procedures. In this context, logical analysis appears to be of great help for the overall epidemiological understanding of latent herpesvirus infections of domestic animals.

It is now obvious that emphasis should be laid more on improved prophylactic methods than on hygienic measures, for the control of herpesvirus infections of domestic animals; this is true not only for economical and ethical reasons but also because the features of latent infections are too often a cause of failure of hygienic measures. It must be stressed however that improved prophylactic methods do not discard the need for good therapeutic tools in the case of clinical disease outbreaks. Among the available drugs, interferon seems to be one of the most promising, even if parenterally administered human interferon α_2 (HuIfnα_2) failed to protect cattle against IBR infection. Further research is still needed in the field of antiviral drugs for veterinary use.

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