Reactivation of Temperature-Sensitive and Non-Temperature-Sensitive Infectious Bovine Rhinotracheitis Vaccine Virus with Dexamethasone

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Latent infections by a temperature-sensitive (ts) infectious bovine rhinotracheitis virus vaccine was produced as frequently as by non-ts vaccine virus. Thus, virus could be reactivated in seven of eight ts vaccinates and six of eight non-ts vaccinates after dexamethasone treatment. Virus excretion could be detectable for 1 to 8 days at a level of $2 \times 10^6$ to $3 \times 10^6$ plaque-forming units per ml of nasal secretions. The reactivated virus was shown to be the same as the original virus used for vaccination by its inability to grow at the restrictive temperature (39°C) as well as by its reactivation endonuclease cleavage pattern.

Like most herpesviruses, infectious bovine rhinotracheitis (IBR) has been shown to induce latency after a primary infection (4, 6, 12). Although the actual site of IBR latency and the mechanism(s) involved in reactivating the virus are not fully understood, it is believed that this virus, like other herpesviruses, can remain latent in neurological cells (19). Since the virus can be readily reactivated after dexamethasone treatment (4, 10, 16, 18; P. O. Pastoret, A. Aguilar-Sefieu, G. Burtonboy, J. Mayer, P. Jettem, and F. Schoenaers, Vet. Microbiol., in press), it is assumed that the immune response is involved either in maintaining the virus in latent state or at least in limiting the amount of virus reexcretion after reactivation.

The extensive use of live attenuated IBR vaccines for controlling IBR infections in cattle (100 × 10⁶ doses in the last 5 years) has raised some concern as to the safety of such vaccines, especially if they themselves produce latency and later revert to virulence. Unfortunately, no one has definitively shown that attenuated IBR vaccines become latent. The main reason for the lack of such definitive proof was that no viral markers were available to differentiate field strains from vaccine strains. The recent development of temperature-sensitive (ts) IBR vaccine (23) and restriction endonuclease fingerprinting techniques has provided us with such markers (17). Using these techniques we have attempted first to determine whether vaccine virus can induce latency and can be reactivated. Second, we wish to determine whether ts vaccines can prevent establishment of infection with virulent field strains. Thus, the ideal vaccine would be one that does not induce latency and also prevents establishment of a latent infection with wild-type virus. The best possible candidate for such a vaccine appears to be appropriate ts vaccines, since theoretically they would be restricted to the surface epithelial cells of the upper respiratory tract and never get established in the neurological cells.

The present report describes our attempts to reconvert ts and non-ts IBR vaccine from animals by the use of dexamethasone, as well as to assess various immune parameters after dexamethasone treatment in an attempt to understand the mechanisms whereby the immune response can prevent reexcretion of virus in nature.

MATERIALS AND METHODS

Cells and virus. Georgia bovine kidney cells were cultured in Eagle minimal essential medium containing 10% fetal bovine serum as described previously (1). Each liter was supplemented with 2 mM glutamine (no. 503, GIBCO Laboratories, Grand Island, N.Y., 25 mg of gentamicin (Schering Diagnostics), 2.5 g of sodium bicarbonate, and nonessential amino acids (no. 114, GIBCO). ts IBR virus vaccine (Norden) and non-ts vaccine virus (Connaught) were cultured from commercial vaccines by infecting confluent GBK monolayers with 1 ml of the reconstituted vaccine, and the virus was allowed to absorb for 1 h before the addition of fresh minimum essential medium-5% fetal bovine serum. The ts vaccine-infected cultures were incubated at 35°C, and the Connaught vaccine (Con-IBR) was cultured at 37°C until they exhibited extensive cytopathology. All the cells and the culture fluids were harvested and subjected to two freeze-thaw cycles before removal of cellular debris by centrifugation (1,000 × g for 10 min). This cell-free preparation was used to

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vaccinate animals as described below. Strain 108, a virulent IBR isolate used for challenge experiments, was obtained from C. Darcel, le Q, Animal Disease Research Institute, Lethbridge, Alberta. It was passaged 2× in our laboratory as previously described (13).

**Animals.** Nine-month-old, healthy, male and female Hereford cattle, free of IBR virus-neutralizing antibody, were randomly divided into two groups of eight animals each (Fig. 1). One group was vaccinated intranasally with *ts* IBR and the other group was vaccinated with Con-IBR (*5 × 10^8* plaque-forming units per animal).

Five and six weeks later, nasal swabs and blood samples were collected from all animals for serum neutralization, antibody-dependent cell cytotoxicity, and blastogenesis assays to obtain a background level of antiviral activity as well as for a hematological profile of each animal (3, 13, 15, 21). Animals were then treated for 5 consecutive days with dexamethasone (0.1 mg/kg of body weight). During the last 3 days of dexamethasone injection, the animals were given an intramuscular injection of antibiotics (Pen-Strep Roger/STB at recommended doses) to prevent possible septicemia. Nasal swabs were obtained daily for virus isolation, and blood samples were collected for hematological and immunological studies (Fig. 1).

Three weeks after the end of the first dexamethasone treatment, the *ts* group was challenged with virulent 108 virus and the Con-IBR group was treated with dexamethasone as described above. Both groups were once again monitored for virus excretion and immunological responses. Four weeks after challenge with virulent 108 virus, the *ts* IBR group was once again treated with dexamethasone and monitored for virus excretion and immunological responses (Fig. 1).

**Virus isolation.** Virus was eluted from nasal swabs (Falcon no. 2009) by blending the swab in a Vortex mixer in a 10-fold volume of minimum essential medium. The amount of virus present in each sample was quantitated by plaqueing 10-fold dilutions of each sample in microtiter plates as described previously (3).

**Neutralization and antibody-dependent cell cytotoxicity assays.** Antibody levels in nasal secretions and serum or plasma were determined by standard neutralization assays as described previously (3). Antibody-dependent cell cytotoxicity assays were performed as described previously (14). The target cells were IBR-infected ^3Cr-labeled GBK cells, and the effector cells were bovine mammary gland polymorphonuclear cells (22). The polymorphonuclear-to-target cell ratio was 50:1.

**Blastogenesis.** Peripheral blood lymphocytes were prepared as described previously (14) and incubated in the presence of ultraviolet-irradiated IBR antigen for 96 h before measuring the level of stimulation (13).

**Hematological studies.** Blood was collected into vacuum tubes (Venoject, KT) containing ethylenediaminetetraacetic acid (EDTA). Blood smears were prepared within 1 h of collection and stained with Wright Giemsa in an Ames-Tek automatic staining machine. Differential blood counts were performed by two different investigators. Other parameters (packed cell volume, hemoglobin, erythrocytes, leukocytes, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration) were determined as described previously (3).

Day 1:

**Group 1 (8 animals)**
Vaccinated with *ts* IBR

**Group 2 (8 animals)**
Vaccinated with Con-IBR

**FIRST DEXAMETHASONE TREATMENT**

Hematological studies, virus isolation

Immunological studies

**CHALLENGE WITH VIRULENT**

Virus (strain 108)

Day 66:

**SECOND DEXAMETHASONE TREATMENT**

Virus isolation

Hematological and immunological studies

Day 94–101:

**SECOND DEXAMETHASONE TREATMENT**

Virus isolation, hematological and immunological studies

Fig. 1. Experimental design.
were assessed in a model S Coulter Counter equipped with appropriate attachments.

Restriction endonuclease analysis of IBR isolates. Viral deoxyribonucleic acid (DNA) was purified by the technique of Pigatti et al. (11). GBK cells in 100-mm-diameter petri dishes were infected with virus and incubated at 35°C. When a majority of cells in the culture displayed cytopathology, the cells were collected, washed 2× with phosphate-buffered saline (PBS) and frozen at −70°C. To extract DNA, the cells were thawed and suspended in 1.8 ml of lysing solution [0.25% Triton X-100, 10 mM EDTA, and 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.9]. After standing at room temperature for 10 min with gentle mixing, NaCl was added to a final concentration of 200 mM. The tube was inverted a few times and centrifuged at 1,000 × g at 4°C for 10 min. DNA in the supernatant was deproteinized by incubating overnight with pronase (100 µg/ml) and sodium dodecyl sulfate (0.2%) followed by extraction with phenol and chloroform (9). DNA was then precipitated twice with ethanol and dissolved in 200 µl of 10 mM Tris (pH 7.8)–10 mM NaCl-0.5 mM EDTA. The DNA solution (75 µl) was then digested with the restriction endonucleases HindIII or EcoRI. For EcoRI, 50 µl of reaction mixture contained viral DNA, 100 mM Tris (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, and 5 U of EcoRI. The reaction mixture for HindIII was comprised of viral DNA, 50 mM NaCl, 6 mM Tris (pH 7.5), 6 mM MgCl₂, 0.1 mg of bovine serum albumin per ml, and 8 U of HindIII. After incubating for 1 h at 37°C, the reaction was terminated by the addition of 50 µl of BJ (100 mM EDTA, 5% sodium dodecyl sulfate, 25% glycerol, and 0.025% bromophenol blue) and heating at 65°C for 10 min.

A 50-µl amount of DNA samples was loaded onto gels in a horizontal 0.5% agarose slab and electrophoresed for 3 h at 150 V. The electrophoresis buffer contained 90 mM Tris (pH 7.9), 90 mM boric acid, and 2.5 mM EDTA. After staining gels with 4 µg of ethidium bromide per ml of electrophoresis buffer, gels were illuminated with an ultraviolet light and photographed with a Polaroid MP4 camera with Polaroid Ty55 P/N film.

RESULTS

Reactivation of IBR virus with dexamethasone and characterization of reactivated virus. After the first dexamethasone treatment, six of eight Con-IBR animals and seven of eight ts IBR animals excreted virus. However, all animals were probably latently infected and reexcreted virus, since all animals exhibited a rise in specific anti-IBR antibody levels. Furthermore, animals that did not excrete detectable virus after the first dexamethasone treatment did so after a second treatment of dexamethasone. In animals where virus excretion was detectable, virus excretion began on the last day of dexamethasone treatment (5 days after initiation of treatment) and lasted for up to 8 days. Although some animals in both groups shed up to 8 logs of virus per ml of nasal secretions, the ts IBR-vaccinated animals on the average excreted more virus (5.25 versus 3.75 logs) over a longer period of time, suggesting that ts IBR vaccines produced latency as readily as did regular vaccines (Table 1).

In an attempt to prove that the excreted virus was ts and not a field strain of virus to which the animals could have been exposed accidentally, we tested the excreted virus for its ability to grow at 39°C. Table 2 illustrates that the virus isolated from the ts IBR-vaccinated animals was indeed ts, since the plaquing efficiency was very low at 39°C.

To confirm that the virus isolated for the ts IBR-vaccinated animals was indeed the ts genotype, we exploited the technique of endonuclease cleavage mapping. Electrophoresis after cleavage with the enzyme EcoRI separates the IBR isoforms.

### Table 1. Reactivation of IBR virus by dexamethasone treatment

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>No. of animals excreting</th>
<th>Primary excretion level</th>
<th>Length of excretion (days)</th>
<th>Secondary excretion level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con-IBR</td>
<td>6/8</td>
<td>2 × 10⁻⁵–3 × 10⁻³</td>
<td>2–5</td>
<td>2 × 10⁻⁵–1 × 10⁻³</td>
</tr>
<tr>
<td>ts IBR</td>
<td>7/8</td>
<td>3 × 10⁻⁵–2 × 10⁻³</td>
<td>1–8</td>
<td>(2.6)</td>
</tr>
</tbody>
</table>

* The level of IBR excretion (plaque-forming units per milliliter of nasal secretions) after the first series of dexamethasone treatments.
* The level of IBR excretion (plaque-forming units of nasal secretions) after the second series of dexamethasone treatments.

### Table 2. Properties of dexamethasone-reactivated IBR virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus yield</th>
<th>34°C</th>
<th>39°C</th>
<th>EOP (39°C/34°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.7 × 10⁴</td>
<td>2.1 × 10⁴</td>
<td>&gt;1</td>
<td></td>
</tr>
<tr>
<td>Con-IBR vaccine</td>
<td>6 × 10⁴</td>
<td>1.8 × 10⁴</td>
<td>&gt;1</td>
<td></td>
</tr>
<tr>
<td>ts IBR vaccine</td>
<td>3 × 10⁴</td>
<td>1.2 × 10⁴</td>
<td>0.4 × 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>Reactivated Con-IBR</td>
<td>4 × 10⁴</td>
<td>1.2 × 10⁴</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Reactivated ts IBR</td>
<td>3.5 × 10⁴</td>
<td>2 × 10⁴</td>
<td>0.57 × 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>Animal no. 4</td>
<td>1.1 × 10⁵</td>
<td>&lt;10⁻⁴</td>
<td>&lt;10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>Animal no. 18</td>
<td>2.25 × 10⁴</td>
<td>&lt;10⁻⁴</td>
<td>&lt;10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>Animal no. 23</td>
<td>8.5 × 10⁴</td>
<td>&lt;10⁻⁴</td>
<td>&lt;10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>Animal no. 25</td>
<td>3.5 × 10⁵</td>
<td>&lt;10⁻⁴</td>
<td>&lt;10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>Animal no. 27</td>
<td>8.5 × 10⁵</td>
<td>&lt;10⁻⁴</td>
<td>&lt;10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>Animal no. 28</td>
<td>4 × 10⁵</td>
<td>&lt;10⁻⁴</td>
<td>&lt;10⁻⁴</td>
<td></td>
</tr>
</tbody>
</table>

* Confluent GBK cells were infected with various IBR isolates at a multiplicity of infection of 0.1. One group of cultures was incubated at 34°C, and the other was incubated at 39°C. Twenty-four hours later the cultures were harvested, and the yield of virus (plaque-forming units per milliliter) was determined. Titration was at 34°C.
* Efficiency of plating (EOP) at 39°C versus 34°C was determined by dividing the virus yield at 39°C by the virus produced at 34°C.
DNA from ts IBR into seven bands which range from 35 to 4.5 megadaltons (Table 3). Each band appears to comprise one fragment, and the fragments are present in the digest in unimolar quantities. In contrast, Con-IBR yields six bands, band C + D being a composite of two fragments. The enzyme HindIII generates 12 bands from ts IBR. Bands C and D are present in 0.5 M amounts, band F + G comprises two fragments, and the remaining bands appear to be unimolar. The HindIII digest of Con-IBR differs from IBR (ts) in that band I is replaced by a lower-molecular-weight band K. In all cases the endonuclease cleavage pattern of the ts reactivated virus was similar to the original ts vaccine, whereas the Con-IBR vaccine virus and virus reactivated from Con-IBR-vaccinated animals were different from the ts IBR (Fig. 2).

Table 3. Molecular weights of restriction endonuclease digests of ts IBR and Con-IBR

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fragments</th>
<th>Con-IBR</th>
<th>ts IBR</th>
<th>Mol wt (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>A</td>
<td>A</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>B</td>
<td>13.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>C</td>
<td>11.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C+D</td>
<td>D</td>
<td>11.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>E</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>F</td>
<td>9.69</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>G</td>
<td>6.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>H</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>I</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>J</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>K</td>
<td>1.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>L</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>M</td>
<td>0.56</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Immune status of animals before and after reactivation. Since dexamethasone treatment reactivated virus, attempts were made at determining whether such treatment altered the level of immunity to IBR virus. In both groups of animals, serum-neutralizing antibodies and target cell-sensitizing antibodies, as detected by virus neutralization and antibody-dependent cell cytotoxicity, were elevated 7 days after termination of dexamethasone treatment (Fig. 3 and 4). These results indirectly confirm that virus excretion occurred.

In an attempt to determine whether ts IBR vaccine could prevent infection with a virulent field strain, the ts IBR-vaccinated animals were infected with a virulent strain (108) 3 weeks after dexamethasone treatment. All eight animals excreted virus for up to 6 days after challenge. These animals exhibited typical IBR lesions in the nasal passages but did not show severe lower respiratory involvement. These animals exhibited a very dramatic increase in the level of immunity after challenge (Fig. 4). If this group was treated with dexamethasone a second time 4 weeks after challenge, no virus excretion could be detected. Furthermore, there was not a significant increase in the level of immunity of the group as a whole, but some individual animals expressed a significant increase. The possible reason for the lack of detectable virus excretion could be that the level of local antibody was of sufficient magnitude that it could neutralize all the virus that was reactivated. Thus, the nasal secretions of all animals could neutralize in excess of 5 logs of virus in vitro.

**DISCUSSION**

Dexamethasone treatment of cattle previously vaccinated with IBR vaccines resulted in a rapid alteration in the number and relative proportions of peripheral blood leukocytes and subsequent reactivation of IBR virus. The frequency of reactivation of virus was the same whether

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**Fig. 2. Electrophoretic analysis of DNA from IBR vaccines; Connaught (C), ts, Norden (TS), and virus isolated after dexamethasone reactivation from three animals (animal no. 4, 19, and 23). Purified DNA samples were digested with the restriction endonucleases EcoRI (E) and HindIII (H). After electrophoresis on 0.5% agarose gels, the gels were stained with ethidium bromide and fluorographed under ultraviolet light. The virus isolates were obtained from nasal secretions of animals that had been vaccinated with ts IBR vaccine and treated with dexamethasone 40 days later, as shown in Fig. 1.**
animals were vaccinated with ts IBR or non-ts Con-IBR (Table 1). These results suggest that both types of vaccines can establish latency with the same frequency. Furthermore, the high frequency of reactivation (80 to 100%) with the vaccines suggests that IBR vaccines are as good at inducing latent infections as is virulent virus. In an attempt to prove that the virus excreted was indeed vaccine virus and not field strains of virus to which the animals were accidently exposed, we used two approaches. First, the viruses isolated from animals were tested for their ability to grow at restricted temperatures (39°C). In all cases the virus reactivated from ts IBR-vaccinated animal was ts, whereas the virus isolated from Con-IBR-vaccinated animals was not ts (Table 2). Secondly, the endonuclease cleavage maps of the reactivated virus matched the endonuclease cleavage maps of the vaccine used to vaccinate the animals. The latter findings suggest that the virus that induces latency and is later reactivated is not a mutant but is representative of the general population used to infect the animal. Whether its genotype and virulence changes with repeated reactivation or whether recombination can occur with virulent field strains was not possible to determine, since virus could not be reactivated after infection of ts-vaccinated animals with virulent virus. The lack of ability to reactivate virulent or ts virus from these animals could possibly be due to the high level of immunity to IBR virus. Since it is postulated that the immune response is not only involved in recovery but also is maintaining herpesvirus latency (2, 4, 7, 20), it is possible that if the level of immunity decreased with time, reactivation and reexcretion may occur. If this occurs, then it should be possible to determine whether recombination between vaccine virus and field strains occurs.

Even though the mean titer of virus excretion by Con-IBR-vaccinated animals was slightly lower (3.75 logs) than ts IBR-vaccinated animals (5.25 logs), some animals in both groups excreted in excess of 8 logs of virus per ml of nasal secretions. The possible reason for the slight increase in the mean level of excretion in the ts IBR animals is that these animals had a lower level of immunity after primary vaccination. The observation that the animals which excreted the highest level of virus were the ones that had the lowest immune response supports the suggestion that latency and reactivation are influenced by the immune status of the animal. Further support for the suggestion that the immune response influenced the level and length of excretion was the observation that after the first episode of excretion the immune response was elevated. Thus, the amount of replication and excretion was limited after a second dexamethasone treatment such that not only was there less virus excreted (2.6 logs) but it was excreted for a much shorter time (1.5 days) in Con-IBR animals. If the immune response was boosted...
even higher by infection with virulent virus, no shedding was detectable after the second dexamethasone treatment. Since dexamethasone is not a very long-acting immunosuppressive drug, discontinued use results in a return to normal, and the immune response can then control both intracellular and extracellular spread of virus and shedding stops. Since immunosuppression is short lived, viral antigens present can act as a booster to the immune response, and therefore this would explain the increase in immunity after dexamethasone treatment and virus reactivation. Presumably, after numerous reactivation cycles, the level of local antibodies present is so high that even though reactivation occurs, infectious virus is rapidly neutralized and excretion cannot be detected.

Although our present results illustrate that the presently licensed ts IBR vaccine induces latency, we feel that it should be possible to produce other ts mutants which do not produce latency. Such latency-negative mutants have been described for ts mutants of herpessimplex (5, 8). If such mutants of IBR are found and they can provide adequate immunity, then they would be the ideal ones to use as vaccines to control IBR. Furthermore, an analysis of the ts lesion and its comparison to herpessimplex virus type 1 latency-negative mutants should provide some information as to the genes required for induction of herpessimplex virus latency. Such experiments are presently in progress.

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LITERATURE CITED