[CANCER RESEARCH 43, 5416–5426, November 1983]

Correlation of Alkaline Phosphatase Activity to Normal T-Cell Differentiation and to Radiation Leukemia Virus-induced Preleukemic Cells in the C57BL Mouse Thymus[1]

Gérard Goffinet,[2] Marie-Paule Houben-Defresne, and Jacques Boniver[3]

Laboratory of Pathological Anatomy, Institute of Pathology B.23, University of Liège, 4000 Liège, Belgium

ABSTRACT

Cytochemical methods at the light and electron microscopic level were used to define the pattern of alkaline phosphatase (APase) activity in normal thymus and to study its modifications after inoculation with the thymotropic leukemogenic radiation leukemia virus in correlation with the emergence of preleukemic cells and their thymus dependency. APase was found in numerous lymphoblasts of the fetal thymus. The enzyme was also detected in a few lymphoid blast cells of the normal young adult thymus, which were closely associated with thymic nurse cells. The observed distribution of APase in normal thymus suggests that its expression could be limited to an early stage of the T-cell differentiation pathway.

After inoculation with radiation leukemia virus, APase activity remained normal for almost the entire latency period, during which virus replication spread to the cortex and thymus-dependent preleukemic cells appeared. An important increase in the number of APase-positive cells occurred later, *i.e.*, at the end of the latency period, in nontumoral thymus, which displayed lymphocytic depletion and contained autonomous thymus-independent preleukemic cells. These latter features obviously reflected the malignant transformation of thymus lymphoblasts, which eventually led to the development of the thymic lymphomas.

The results raise the question of the possible filiation between the thymic nurse cell-associated APase-positive lymphoid cells of the normal thymus and the target cells susceptible to productive infection and to neoplastic transformation after radiation leukemia virus infection.

INTRODUCTION

RadLV[4] is a thymotropic retrovirus which induces the development of lymphoblastic lymphomas in the thymus after inoculation into C57BL/Ka mice (7, 8, 34). The thymotropism of RadLV has been explained by the selective presence in the thymus of target cells susceptible to productive infection by the virus (1, 9, 10). These target cells belong to a small subpopulation of cortical lymphoid blast cells at the earliest stage of the intrathymic T-cell differentiation pathway (1). After infection, virus replication starts in lymphoid cells, which are engulfed in the cytoplasmic processes of cortical epithelial cells, *i.e.*, within the so-called TNCs (24); these structures are lymphoepithelial complexes involved in the earliest steps of T-cell differentiation (25, 29, 47). Eventually, virus replication spreads to the entire cortical thymocyte population (1, 10). However, the thymus remains apparently normal for a long latency period of 3 to 6 months. Neoplastic lymphoblasts eventually proliferate in the thymus cortex and give rise to a thymic tumor (10).

Besides morphological studies, little is known about the sequence of events occurring in the thymocyte population between virus infection and neoplastic growth. Several investigations suggest the emergence of potential leukemia-inducing cells, also designated "preleukemic" cells (18, 19), as a necessary transient step in the leukemogenic process. Preleukemic cells are found in mice that do not express any evidence of thymic lymphomas; they are detected by an *in vivo* transplantation assay, which is based on their capacity to give rise to lymphomas after grafting into histocompatible hosts (17). They were observed in macro- or microscopically nontumoral thymuses at various time intervals, either after whole-body irradiation (2) or after inoculation with RadLV (23) or with the A-RadLV variant (18). Preleukemic cells were also found in bone marrow of C57BL/6 mice after infection by the D-RadLV variant or after irradiation (18). The same is also true concerning AKR mice, which develop spontaneous lymphomas (19, 21). These reports indicate that preleukemic cells first require thymic environment for progressing to frank neoplasia and eventually become autonomous (18–20).

Very little information is available on the characterization of preleukemic cells. Haran-Ghera *et al.* (19, 20) reported that, in their experimental model, bone marrow preleukemic cells belong to the population of prothymocytes. And, virtually nothing is known about those which are first found in the thymus. In particular, no correlation has been made thus far between the appearance of preleukemic cells and the expression of a "tumor marker."

The present work was undertaken in an attempt to establish such a correlation in C57BL/Ka mice after inoculation with RadLV. The putative "tumor marker" used in this study was membrane APase. The enzyme activity was analyzed in conjunction with the emergence of preleukemic cells and their dependency upon the thymic microenvironment. The choice of APase as a candidate tumor marker was based on the previous detection of the enzyme by histochemical methods at the level of light microscopy in spontaneous lymphomas of AKR mice (43) and in radiation or RadLV-induced lymphomas of C57BL mice (31, 41, 44). The enzyme might be an oncofetal marker of T-lymphocytes in mice since it was also described in fetal thymuses (31) but not in normal adult thymuses or during the latency period preceding the onset of lymphomas (31).

[1] These studies were supported in part by the Fonds de la Recherche Scientifique Médicale and the Centre Anticancéreux près l'Université de Liège.

[2] Present address: Institute of Zoology, 22, Quai Van Beneden, University of Liège, 4020 Liège, Belgium.

[3] Chercheur Qualifié du Fonds National de la Recherche Scientifique. To whom requests for reprints should be addressed.

[4] The abbreviations used are: RadLV, radiation leukemia virus; TNC, thymic nurse cell, APase, alkaline phosphatase; PBS, phosphate-buffered saline (0.85% NaCl solution, containing per liter 2.15 g Na_2HPO_4 and 0.54 g KH_2PO4, pH 7.2); FCS, fetal calf serum; TdT, terminal deoxynucleotidyl transferase.

Received October 27, 1982; accepted August 5, 1983.

MATERIALS AND METHODS

Animals. Four-week-old C57BL/Ka mice of both sexes were used in most studies; some experiments were performed with 16- to 18-day-old fetuses of the same mouse strain. Congenic (Thy,1-1) C57BL/Ka mice (called BL/1.1 for convenience), which were developed by M. Lieberman at Stanford University, were used in the transplantation experiments for the detection of preleukemic thymocytes (see below).

Virus. The thymotropic (T^+), leukemogenic (L^+) RadLV/VL3 virus was obtained from the culture fluid supernatant of BL/VL3 cells. This cell line was established from a RadLV-induced thymic lymphoma (33). Intrathymic injections were made in both lobes with 0.05 ml of a virus preparation with a titer of 10^7 infectious particles/ml, as estimated by an *in vitro* immunofluorescence assay (32). This virus isolate induced thymic lymphomas in 90 to 100% of the treated mice after a 3- to 9-month latency period. Intrathymic inoculation of 0.05 ml of PBS was done in age-matched control mice; these mice did not develop any detectable disease.

Isolation of TNCs. TNCs were obtained by using a slight modification of the method originally described by Wekerle and Ketelsen (47). The method was described in detail elsewhere (24). Briefly, thymuses were minced with scissors and washed for 10 min in PBS. The remaining fragments were incubated in the presence of Dispase (Dispase II; 1.2 units/ml in PBS; Boehringer Mannheim) and DNase (DNase I; 0.02 mg/ml in PBS; Boehringer Mannheim), using a magnetic stirrer. The enzyme solution was renewed 2 or 3 times. The dissociation was achieved by 2 or 3 incubations in a collagenase solution (Collagenase Worthington CLSIV; 40 units/ml in PBS; Millipore Corp.). TNCs were isolated from the resulting cell suspensions by successive runs of 1 g sedimentation on 30% FCS in PBS as described previously (24).

This method usually yields 15,000 to 20,000 TNCs per thymus of young adult C57BL/Ka mice; after a 20-hr *in vitro* incubation, an average of 10 lymphoid cells was recovered from each TNC; it must be noted, however, that by electron microscopy one can estimate the thymocyte number per TNC to range between 5 and 50. As used by us, the procedure described above was the most efficient for getting the highest number of TNCs from normal thymuses; as shown by monoclonal antibody staining and flow cytometry, the phenotype (*i.e.*, membrane Thy-1, Lyt-1, Lyt-2 antigens) of the lymphocytes recovered after enzyme dissociation does not differ significantly from that of cells which are prepared by mechanical methods.[5]

Histochemistry. For histochemical detection of alkaline phosphatase at the level of light microscopy, thymuses were frozen at −20°, embedded in Tissue Tek II (Lab Tek Products, Miles, Brussels, Belgium), and cut with an IEC CTF microtome cryostat (Jibé; Brussels, Belgium). Five-μm sections were fixed in formaline diluted at 10% in absolute methanol and incubated in the presence of Naphtol AS-MX and Fast Red Blue RR, following a standard procedure.[6] Positive reactions were seen as blue granules on the cell membrane.

Ultrastructural Cytochemistry. For electron microscopy, APase was cytochemically demonstrated by the calcium-lead nitrate method, which was developed by Molnar (37) and adapted by Reale and Luciano (38, 39) for electron microscopy. This method was preferred to others in which lead nitrate is present in the incubation medium together with the substrate. Indeed, under these latter conditions, *i.e.*, when lead salts are used at the same time as the substrate, the incubation medium becomes unstable rapidly. This can result in the formation of important nonspecific deposits. To avoid these artifacts, we used the procedure proposed by Reale and Luciano (38, 39) in which the tissues are exposed to lead nitrate after incubation with the substrate; however, under these conditions, it is possible that the precipitate is translocated because calcium phosphates are soluble in acidic solutions. Therefore, in order to overcome this disadvantage, preliminary investigations were performed on thymus slices and on tissues, which are known to contain APase, such as those of the intestine. These technical studies demonstrated that the diffusion artifacts could be minimized by briefly rinsing the tissues with freshly distilled water, after treatment in the incubation medium. Therefore, the following procedures were used to detect APase by electron microscopy.

Cell suspensions or tissue fragments were first fixed for 30 to 60 min in a solution of 0.1 M sodium cacodylate buffered with 2.5 or 4% glutaraldehyde (pH 7.4). Tissue fragments were then cut into 20- to 40-μm slices with the Oxford Vibratome (Delforge, Brussels, Belgium). They were incubated for 30 to 45 min in a medium (pH 9.5) prepared as follows: 0.2 M $CaCl_2$, 0.1 M sodium glycerophosphate, 0.05 M $MgCl_2$; after being rinsed for 5 min in cold freshly prepared distilled water, they were placed in a cold 0.05 M lead nitrate solution. Negative controls were prepared by adding specific inhibitors such as 1-*p*-bromotetramisole oxalate (0.04 mM) (Aldrich, Beerse, Belgium) or KCN (1 mM) to the incubation medium (4, 11). The specimens were then postfixed in 1% OsO_4 solution for 30 min at 4°. Routine procedures were used for the next steps of the sample processing. In order to facilitate the orientation of thymus pieces, tissue slices were embedded in flat silicone rubber molds.

Detection of Preleukemic Cells. Preleukemic cells were detected by using an *in vivo* assay, modified from a method previously described (2, 17). This test involves intrathymic or i.v. injections of cell suspensions, from RadLV-treated C57BL/Ka mouse tissues into 400-rad-irradiated 2-month-old congenic BL/1.1 mice. C57BL/Ka and BL/1.1 mice differ only by the Thy-1 allele which is expressed on thymocyte and on T-cell membranes (13); indeed, the former express Thy-1.2, while the latter ones express Thy-1.1. The 400-rad dose of whole-body irradiation was given in order to facilitate the proliferation of donor cells in the host thymus (2, 18). Since previous reports indicate that preleukemic cells first require thymic microenvironment for growth to become autonomous (18–20), their "thymus dependency" was tested by inoculating cell suspensions from RadLV-treated donor mice into 2 types of recipients; in the first group, the animals were intact, whereas in the second group, they had been thymectomized 1 month before use. Recipients were sacrificed when moribund or showing clinical evidence of lymphoma. The donor or recipient origin of the tumors found at the autopsy was revealed by immunofluorescence detection of the donor type Thy-1.2 or recipient type Thy-1.1 antigens. It must be noted that it was not possible to test the origin of all lymphomas; indeed, in some cases, mice were found dead and thus were only dissected to ascertain the presence of a tumor; immunofluorescence staining could not be done on necrotic tissues.

This experimental protocol usually yields several types of results, which are interpreted as follows. In come cases, the thymic lymphomas obtained in the recipients were made of host cells and thus were induced by leukemogenic virus particles which were transferred with the injected cell suspension. Other cell inoculates, which led to the development of lymphomas only in thymus-bearing recipients, thus contained "thymus-dependent" preleukemic cells, *i.e.*, cells which required thymic microenvironment to progress to frank lymphoma. Finally, in other cases, lymphomas were observed in both thymus-bearing and thymectomized recipients, indicating that the "thymus-independent preleukemic" cells were present in the inoculate. This interpretation of the results is summarized in Table 1.

In the present experiments, groups of 5 to 10 C57BL/Ka donor mice were sacrificed on Days 15, 30, 45, 60, 75, 90, and 120 after inoculation with RadLV. Other mice were kept for a longer period and sacrificed when they showed symptoms of lymphoma dissemination. Thymuses were removed, weighed, and split into 2 parts. Half of each thymic lobe was used for the cytochemical detection of APase by light and by electron microscopy. The other half was used to prepare cell suspensions in PBS supplemented with 5% FCS. Aliquots of 5×10^6 cells from each donor mouse were inoculated either intrathymically into 2 or 3 400-rad-irradiated thymus-bearing recipients or i.v. into the same number of 400-rad-irradiated thymectomized mice. The animals were then handled as described above.

Membrane Immunofluorescence. To determine the donor or host

[5] M-P. Houben-Defresne, unpublished data.

[6] Sigma Technical Bulletin No. 85, Sigma, Brussels, Belgium.

origin of the tumors found at the autopsy of the recipient mice, tumor cell suspensions were prepared in PBS supplemented with 5% FCS; cells were then incubated with either anti-Thy-1.2 or anti-Thy-1.1 monoclonal antibodies (New England Nuclear, Liège, Belgium) diluted at 1/1000 and then with fluorescein isothiocyanate-conjugated goat anti-mouse IgM (Nordic Laboratory, Leuven, Belgium) at a 1/40 dilution (22). The percentage of Thy-1.2 donor-derived or Thy-1.1 recipient-derived cells was defined with the aid of a fluorescence-activated cell sorter (FACS IV; Becton Dickinson, Sunnyvale, Calif.) (35).

RESULTS

APase in Normal Thymus. In 16- to 18-day-old fetal thymuses, cortical and medullar regions were not yet distinguishable. The major cell population was made up of epithelial cells and of lymphoblasts. Using an electron microscope, the majority of these lymphoid cells exhibited a faint APase positive reaction, seen as electron dense deposits along the outer side of the plasma membrane (Fig. 1). This ultrastructural observation confirmed the previous light microscopic studies in fetal thymuses (31).

In the thymus of 30-day-old young adult mice, APase activity was found in stromal cells of the cortex and of the medulla using the light microscope only. At the ultrastructural level, APase was observed in plasma cells, in endothelial cells, and in nonlymphoid stromal cells (Figs. 2 and 3). Moreover, a few cortical lymphoid cells displayed an enzyme-positive reaction that was not detected by light microscopy. Interestingly, most, if not all, of these cells were in close contact with epithelial cells (Fig. 4).

Table 1

Interpretation of the in vivo transplantation assay for the detection of preleukemic cells

Observation in the thymus-bearing recipients	Observation in the thymectomized recipients	Conclusion
No tumor	No tumor	No leukemogenic agent or preleukemic cell transferred
Thymic lymphoma of recipient origin	No tumor	Transfer of thymotropic, leukemogenic virus particles
Thymic lymphoma of donor origin	No tumor	Transfer of thymus-dependent preleukemic cells
Thymic lymphoma of donor origin	Lymphoma of donor origin	Transfer of thymus-independent preleukemic cells

The association between APase-positive lymphoid cells and cortical epithelial cells led us to investigate the enzyme expression within the TNCs. These lymphoepithelial complexes were isolated from normal young adult mice by enzyme dissociation and 1 g sedimentation, as described in "Material and Methods." The APase procedure for electron microscopy was applied. Twenty % of normal TNCs contained at least one APase-positive lymphoid cell (Figs. 5 and 6), whereas no activity was found on thymocytes which were not associated with epithelial cells. It was demonstrated that APase activity was expressed on the thymocyte membrane itself. Indeed, when lymphoid cells were released from TNCs by mild sonication or by a brief *in vitro* incubation before cytochemical staining, the APase activity was clearly observed on the plasma membranes of the recovered lymphocytes (Fig. 7). Allowing for an average of 10 lymphoid cells/TNC (25), it could be estimated that about 2% of the TNC-associated lymphoid population contains membrane APase.

The addition of KCN or bromotetramisole to the incubation medium strongly inhibited the APase activity of the normal thymus in the fetus and in the adult mice.

APase and "Preleukemic Cells" in the Thymus after Inoculation of RadLV. The morphological modifications induced in the thymus by a local injection of RadLV were documented in detail in previous histological and ultrastructural studies (1, 10). Only the most important features will be described here in connection with the observations on APase activity and preleukemic cells.

Two weeks after inoculation with RadLV, the thymus appeared to be slightly smaller than that in controls. However, the microscopical morphology appeared normal. The majority of cortical lymphoblasts and small lymphoblasts exhibited budding type C virus particles. A few sparse dark epithelial cells were APase positive (Fig. 8). Moreover, a few lymphoblasts, located in the outer cortex, displayed membrane APase activity, which was detected only by electron microscopy (Figs. 9 and 10). Obviously, they were not more significant in number when compared to those in control thymuses (see below). In the same thymic tissues, "thymus-dependent preleukemic" cells were found by the *in vivo* assay. Indeed, inoculation of 400-rad-treated thymus-bearing BL/1.1 recipients with C57BL/Ka thymocytes from donor mice sacrificed 14 days after virus injection gave rise to lymphomas of donor origin in 3 of the 5 mice which were analyzed (Table 2); 2 tumors were derived from host cells; on the contrary, no tumor developed in thymectomized recipients.

Table 2

Preleukemic cells and APase activity in the thymus after inoculation with RadLV[a]

Time after RadLV injection (days)	Thymus wt (mg)[b]	APase activity in the thymus[c]		Lymphomas in thymus-bearing recipients			Lymphomas in thymectomized recipients		
					Origin			Origin	
		Normal	Increased	Incidence	Donor	Recipient	Incidence	Donor	Recipient
15	47.5 ± 4.5[d]	5/5		9/10	3/5 (55)[e]	2/5 (100)	0/10		
30	47.9 ± 4.5	5/5		12/15	6/6 (55)		0/16		
45	45.4 ± 4.65	5/5		10/13	4/6 (85)	2/6	0/15		
75	38.2 ± 10.9	5/5		11/11	(100)		0/16		
90	26.0 ± 12	2/4	2/4	4/8	4/4 (45)		3/6	3/3	
120	39.0 ± 10	1/10	9/10	17/20	13/13 (70)		13/24	10/10	

[a] See "Materials and Methods."
[b] Weight in control mice: 1 month old, 67 mg ± 5; 2 months old, 58.5 mg ± 72; 3 months old, 60 mg ± 5.5.
[c] Defined by cytochemistry at the light and the electron microscope level. Normal, activity similar to that of PBS-inoculated age-matched control mice; increased, foci or diffuse areas with APase-positive lymphoid cells (see text).
[d] Mean ± S.D.
[e] Average latent period in days.

From Day 30 to Day 75, thymuses of virus-infected mice appeared to be very similar to those described on Day 14. Here again, a weak APase activity was detected by electron microscopy and thymus-dependent preleukemic cells were found (Table 2).

The expression of APase and the thymus-dependent characteristics of the preleukemic cells changed drastically from Day 90 onwards. Most of the thymuses collected on Days 90 and 120 were macroscopically normal or atrophic. At the light microscopy level, the general architecture was preserved. Cortex and medulla appeared clearly distinct. The number of small lymphocytes was decreased in foci of various sizes or in more diffuse areas of the cortex. The number of lymphoid blast cells was rather normal but, because of the lymphocytic depletion, epithelial cells and macrophages were prominent. In the depleted areas, numerous lymphoid blast cells contained membrane APase which was detected easily by both light and electron microscopical cytochemistry (Figs. 11 and 13, *c* and *d*). The 10 thymuses in which APase expression was high contained thymus-independent preleukemic cells. Indeed, inoculation of a cell suspension obtained from these thymuses into 400-rad-treated BL/1.1 mice led to the development of lymphomas, whether the hosts were thymectomized or not.

In donor mice, thymic lymphomas were also observed from Day 120 onward. Membrane APase was found on the majority of tumoral lymphoblasts (Figs. 12 and 13, *e* and *f*); inoculation of neoplastic cells into BL/1.1 recipients, regardless of irradiation or thymectomization, resulted in the growth of lymphomas of donor origin after a short latency period of about 30 days (data not shown).

The expression of APase was low in thymuses collected from mice sacrificed as controls at various time intervals after an intrathymic inoculation of PBS (Fig. 13, *a* and *b*). Only a few positive lymphoblasts were found in the outer cortex by electron microscopy. Some other positive nonlymphoid cells, similar to those described in the normal young adult thymus, were also seen (see above). As expected, inoculation of cell suspensions prepared from these thymuses never gave rise to lymphoma in BL/1.1 recipients (data not shown).

The APase activity in the virus-injected thymuses was qualitatively similar to that observed in the fetal and in the young adult thymus: adding KNC or 1-*p*-bromotetramizole to the incubation medium for the ultrastructural cytochemistry inhibited the enzyme activity.

DISCUSSION

Our observations on the expression of APase in the *normal thymus* of fetuses and of young adult mice strongly suggest that the enzyme might be a phenotypical marker of immature T-cells. Indeed, APase was found on the plasma membranes of numerous lymphoblasts in thymuses of 16- to 18-day-old fetuses. This age in fetal life is critical for the ontogeny of the thymic cortex lymphoid cell population (16, 28, 36). The enzyme disappeared almost completely in adult life. Whereas it was found only in nonlymphoid stromal cells by light microscopy, the use of ultrastructural cytochemistry allowed us to detect a few APase-positive lymphoid cells in the thymus of young adult mice. These positive cells were lymphoblasts, which were found to be closely associated with epithelial cells and therefore most likely to be misinterpreted as stromal cells at the light microscopy level. Differences in APase demonstration by electron *versus* light microscopy may also be due to differences in the sensitivity of the 2 substrates for the enzyme; however, this possibility has not been investigated in the present work.

After enzyme dissociation of the thymus, APase-positive cells were selectively recovered in TNCs. Quantitative estimates suggest that the APase-containing lymphoid cells represent at least 2% of the TNC-associated population, *i.e.*, about 2 out of every 100,000 thymocytes (allowing that the TNC-associated lymphoid cell population represents 0.1% of the whole thymus). This observation must be considered with regards to the significance of TNCs and intra-TNC lymphoid cells in T-cell lymphopoiesis.

TNCs are lymphoepithelial complexes, which can be isolated from dissociated thymuses by a simple cell separation procedure of sedimentation at $1 \times g$. The methods of enzyme dissociation and of cell separation used in this study were optimized to obtain the highest yield of TNCs. As seen by electron microscopy (Fig. 5) (47, 48), each TNC results from the apparent "engulfment" of thymocytes within the cytoplasmic processes of large stromal cells. The epithelial nature of this stromal cell was established by the presence of cytoplasmic tonofilaments (47, 48) and by an intense immunofluorescence staining with antibody to keratin (45). The question of the real existence of TNCs *in vivo* has been considered by several authors. Indeed, as mentioned by Ritter *et al.* (40), one could imagine that TNCs might be artifacts resulting either from cell damage during enzyme dissociation or from endocytosis of lymphocytes during cell suspension preparation. Several arguments are clearly against this hypothesis. TNCs can be routinely obtained by mechanical dissociation (40, 48), thereby excluding the involvement of enzyme damage. Moreover, experiments reported by Kyewski and Kaplan (29) nicely demonstrated that the envelopment of thymocytes indeed resulted from a specific *in vivo* association and not from a random attachment of thymocytes to the epithelial cells during the isolation procedure. Final and definitive clues in favor of the existence of TNCs *in vivo* are their identification in the thymus itself. Indeed, Ritter *et al.* (40) identified TNCs in carefully processed frozen sections of human thymus. More recently, examination of cryofractures of mouse thymus with scanning electron microscopy allowed us to detect TNCs in the outer cortex.[7] It must also be noted that careful observation of thin section of the thymus subcapsular zone with a transmission electron microscope revealed a close association between thymocytes and epithelial cells. These observations clearly indicate that TNCs represent a reproducible *in vitro* correlate of an *in vivo* lymphoepithelial complex.

It is most likely that TNCs contained a particular subset of thymocytes engaged in the earliest steps of intrathymic lymphopoiesis. (*a*) Most TNCs derive from the subcapsular zone (24, 29) where thymocyte proliferation takes place (46). (*b*) Immunofluorescence studies on permeabilized TNCs or on lymphoid cells released from TNCs after collagenase- (29) or dispase-collagenase dissociation demonstrated that most, if not all, of the intra-TNC thymocytes display the Thy-1+, Lyt-1+, Lyt-2+, PNA+ phenotype and contain cytoplasmic TdT. In a few cells, intranuclear TdT was also detected (25). An important set of observations indicate that the presence of immature thymocytes within TNCs relate to critical stages of the intrathymic T-cell differentia-

[7] M. P. Houben-Defresne, G. Goffinet, and J. Boniver, *In situ* identification of thymic nurse cells in the mouse thymus, manuscript in preparation.

tion pathway. Indeed, studies which were devoted to murine and human TNCs demonstrated that their epithelial component expressed high levels of membrane major histocompatibility complex products, thus suggesting that TNCs might be involved in the acquisition of self-recognition by immature thymocytes (40, 47, 48). More recently, studies from our laboratory[8] and from others (29, 30) on TNC restoration after sublethal irradiation and bone marrow grafting support the view that TNCs contain some immature cells belonging to the early progeny of bone marrow-derived or of intrathymic T-cell precursors. It is tempting to postulate that the rare APase-positive lymphoid cells demonstrated here within TNCs correspond to this subset. It is worthwhile to mention that, in bone marrow, some medium-sized lymphoid cells of low-density display both membrane APase activity and nuclear TdT;[9] the ultrastructural features of these cells are similar to those of the TdT-positive bone marrow cells (15), which were described as T-cell progenitors (42). Further studies are now in progress to define more accurately the phenotype of the APase-positive cells and to determine the possible role of the enzyme in the early steps of T-lymphopoiesis.

The relation of APase activity to the emergence of preleukemic cells after a leukemogenic inoculation with RadLV had not been investigated before. During a long latent period, APase activity was low and restricted to rare cortical lymphoid blast cells, which were detected by electron microscopy. Meanwhile, "thymus-dependent" preleukemic cells were found. Later, during the fourth month after the leukemogenic treatment, an increase in the number of APase-positive cells was observed in preleukemic thymuses which was obviously related to the appearance of the so-called thymus-independent preleukemic cells. Later on, as observed previously by Lagerlof and Kaplan (31), all thymic lymphomas induced in the C57BL/Ka mouse strain were APase positive.

These data, together with the results of previous investigations, yield new insights into the sequence of events occurring during the malignant process induced by RadLV. First of all, at the time of virus infection, there is a specific interaction between infectious virus particles and a small subpopulation of cortical thymocytes (1, 10, 27). The infection of target cells by RadLV is followed by virus replication which first occurs in lymphoid blast cells of the thymic subcapsular zone and then spreads to the entire cortical population (1, 10). Meanwhile, thymus-dependent preleukemic cells appear in the thymus 1 or 2 weeks before their emergence in bone marrow (23). Correspondingly, the APase activity remained very weak and could be seen in thymocytes only by electron microscopy. Interestingly enough, the first virus-producing cells and the first preleukemic cells were both located preferentially within TNCs (23, 24), in which we found most of the APase-positive thymocytes. Thus, one might propose that target cells for RadLV belong to a unique subpopulation of immature thymocytes which exhibit APase activity and are engulfed within TNCs. Cell-sorting experiments based on the expression of membrane APase would certainly be helpful to test this hypothesis. For this purpose, the production of anti-APase monoclonal antibodies is now in progress in our laboratory. The adaptation of APase cytochemistry for living cell analysis by flow fluorometry (3, 11) would also contribute to the resolution of this critical question.

Thymus-dependent preleukemic cells are clearly distinct from normal thymocytes by their cell kinetics. They can last for more than 2 weeks in the thymus since they give rise to a lymphoma after a latent period of 2 or 3 months. On the contrary, the survival of transplanted normal thymocytes never exceeds 2 or 3 weeks (26). However, the thymus-dependent preleukemic cells do not show the proliferative capacities of lymphomatous cells; if so, they would have been expected to give rise to a thymic tumor in about 1 month or less (this paper and Ref. 33); as mentioned above, the average latency period before lymphoma growth in recipients of preleukemic cells is about 2 months or more. Furthermore, the thymus-dependent preleukemic cells are not autonomous; they require a period of residence (*i.e.*, the latency period preceding lymphoma growth) in the thymic environment to evolve into frank neoplasia. This dependent neoplastic potential was also reported in the studies of Haran Ghera (18–20) on preleukemic cells.

Two or 3 months after inoculation of the virus, thymus-independent preleukemic cells were found in atrophic or macroscopically normal thymuses. Rather numerous APase positive cells were also observed in these thymuses. Later on, in frank neoplasia, the lymphomatous cells exhibited membrane APase. Similar features were also observed in the preleukemic atrophic thymuses from mice sacrificed 3 or 4 months after a leukemogenic course of whole-body irradiation (2).[10]

Taken together, the observations suggest that lymphocyte depletion, APase expression in lymphoblasts, and thymus-independent growth capacities (*i.e.*, autonomy) are somehow related to the final events leading to lymphoma transformation in the thymus. However, our data do not establish whether the thymus-independent preleukemic cells described in this study are already fully neoplastic or whether they require some further change to express the frank cancerous phenotype. Moreover, it has still to be demonstrated whether the neoplastic cells belong to the progeny of target cells for productive infection by RadLV and/or to that of the thymus-dependent preleukemic cells. Here again, cell-sorting experiments based on the detection of APase will be helpful.

In our experimental material, APase activity was found in all the RadLV-induced thymic lymphomas that we tested by cytochemical methods. As mentioned above, these observations are in agreement with the data reported previously by Lagerlof and Kaplan (31), who used the same mouse strain. However, they are in contrast with a recent report, in which the authors describe only low levels of APase activity, if any, in murine T-cell lymphoma cell lines (6). It must be noted that the tumors tested in these studies were cell lines established *in vitro*. Recently, we observed a dramatic loss of APase activity after culturing lymphomas cells, which were initially APase positive, for a long time.[10] However, this phenomenon was not seen in all the cell lines that we established. In any case, it thus appears that, unlike the phosphokinase found in the tumors induced by the Rous sarcoma virus (5), the expression of APase is not required for maintaining the neoplastic state in T-cell lymphomas. The reason why APase can disappear from cell lines established *in vitro* has still to be analyzed.

Thus, APase is expressed in a few normal immature T-cells,

[8] M. P. Houben-Defresne, A. Varlet, and J. Boniver, Thymic nurse cells and thymic repopulation after whole body sublethal irradiation in mice, submitted for publication.

[9] P. Lenaerts, and J. Boniver, Characterization of alkaline phosphatase positive lymphoid cells in the normal C57BL mouse bone marrow, manuscript in preparation.

[10] J. Boniver, P. Lenaerts, and M. P. Houben-Defresne, unpublished data.

increases after RadLV inoculation together with the emergence of thymus-independent preleukemic cells, and is found in most thymic lymphoma cells. We therefore propose that the enzyme may be used as a marker in the studies on T-cell lymphopoiesis and leukemogenesis. Their presence in lymphoma cells and probably in thymus-independent preleukemic cells might indicate that APase-positive immature cells of the T-lineage act as target cells for the oncogenic effect of RadLV and thus finally give rise to a tumor bearing the same membrane marker. Alternatively, the enzyme might be only "reexpressed" in neoplastic cells, in the same way as oncofetal antigens. It must be mentioned, however, that APase is not restricted to the early stages of the T-cell lineage and to T-cell lymphomas. Indeed, it was recently observed that high levels of APase activity are present in some pre-B- and B-cell lymphomas (6), in activated normal B cells (14), and in erythroid cells of postirradiation regenerating bone marrow.[9] APase might thus be expressed in several hemopoietic cell lineages, but in all cases it appears to be closely related to the proliferation and differentiation process and thus might be used as a marker in those cell lineages. The function of the enzyme in the hemopoietic system is still unknown and thus requires further investigation.

ACKNOWLEDGMENTS

We are indebted to Dr. H. S. Kaplan and M. Lieberman for providing the C57BL/Ka mouse strains and BL/VL3 lymphoma cell line. We are grateful to Professor E. H. Betz and to Dr. B. Yellowe for reviewing the manuscript. We thank C. Demoulin and G. Righetti for their excellent technical assistance and A. Lisin and A. M. Valkenborgh for secretarial assistance.

REFERENCES

1. Boniver, J., Decleve, A., Honsik, C., Lieberman, M., and Kaplan, H. S. Phenotypic characterization of thymus target cells susceptible to productive infection by the radiation leukemia virus. J. Natl. Cancer Inst., *67:* 1139–1151, 1981.
2. Boniver, J., Decleve, A., Lieberman, M., Honsik, C., Travis, M., and Kaplan, H. S. Marrow-thymus interaction during radiation leukemogenesis in C57BL/Ka mice. Cancer Res., *41:* 390–392, 1981.
3. Boniver, J., Lenaerts, P., and Houben-Defresne, M. P. Détection cytochimique et analyse cytofluorimétrique des phosphatases alcalines dans des lignées continues de lymphomes thymiques induits chez la souris par le virus des ratioleucoses. C. R. Soc. Biol., *176:* 568–574, 1982.
4. Borgers, M., and Thone, F. The inhibition of alkaline phosphatase by 1-*p*-bromotetramisole. Histochemistry, *44:* 277–280, 1975.
5. Brugge, J. S., and Erikson, R. L. Identification of a transformation specific antigen induced by an avian sarcoma virus. Nature (Lond.), *269:* 346–348, 1977.
6. Culvenor, J. G., Harris, A. W., Mandel, T. E., Whitelaw, A., and Ferber, E. Alkaline phosphatase in hemotopoietic tumor cell lines of the mouse: high activity in cells of the B lymphoid lineage. J. Immunol., *126:* 1974–1977, 1982.
7. Decleve, A., Lieberman, M., Ihle, J. N., and Kaplan, H. S. Biological and serological characterization of radiation leukemia virus. Proc. Natl. Acad. Sci. U. S. A., *73:* 4675–4679, 1976.
8. Decleve, A., Lieberman, M., Ihle, J. N., Rosenthal, P. N., Lung, M. L., and Kaplan, H. S. Physicochemical, biological and serological properties of a leukemogenic virus isolated from cultured RadLV-induced lymphomas of C57BL/Ka mice. Virology, *90:* 23–35, 1978.
9. Decleve, A., Sato, C., Lieberman, M., and Kaplan, H. S. Selective thymic localization of murine leukemia virus related antigen in C57BL/Ka mice after inoculation with radiation leukemia virus. Proc. Natl. Acad. Sci. U. S. A., *71:* 3124–3128, 1974.
10. Decleve, A., Travis, M., Weissman, I. L., Lieberman, M., and Kaplan, H. S. Focal infection and transformation *in situ* of thymus cell subclasses by a thymotropic murine leukemia virus. Cancer Res., *35:* 3585–3595, 1975.
11. Dolbeare, F., Vanderlaan, H., and Phares, W. Alkaline phosphatase and an acid acrylamidase as marker enzymes for normal transformed WI-38 cells. J. Histochem. Cytochem., *28:* 419–426, 1980.
12. Doty, S. B. Problems inherent in obtaining the alkaline phosphatase reaction. J. Histochem. Cytochem., *28:* 66–68, 1980.
13. Finn, O. J., Boniver, J., and Kaplan, H. S. Induction, establishment *in vitro* and characterization of functional antigen specific, carrier-primed murine T-cell lymphomas. Proc. Natl. Acad. Sci. U. S. A., *76:* 4033–4037, 1979.
14. Garcia-Rozas, C., Plaza, A., Diaz-Espada, F., Kreisler, M., and Martinez-Alonso, C. Alkaline phosphatase activity as a membrane marker for activated B cells. J. Immunol., *129:* 52–55, 1982.
15. Goldschneider, I., Metcalf, D., Mandel, T., and Bollum, F. J. Analysis of rat hemopoietic cells on the fluorescence-activated cell sorter. II. Isolation of terminal deoxynucleotidyl transferase-positive cells. J. Exp. Med., *152:* 438–445, 1980.
16. Gregoire, K. E., Goldschneider, I., Barton, R. M., and Bollum, F. Ontogeny of terminal deoxynucleotidyl transferase positive cells in lymphohemopoietic tissues of rat and mouse. J. Immunol., *123:* 1347–1352, 1974.
17. Haran-Ghera, N. Relationship between tumor cell and host in chemical leukemogenesis. Nat. New Biol., *246:* 84–86, 1973.
18. Haran-Ghera, N. Spontaneous and induced preleukemia cells in C57BL/6 mice: brief communication. J. Natl. Cancer Inst., *60:* 707–710, 1978.
19. Haran-Ghera, N. Potential leukemic cells among bone marrow cells of young AKR/J mice. Proc. Natl. Acad. Sci. U. S. A., *77:* 2923–2926, 1980.
20. Haran-Ghera, N., Rubio, N., Leef, F., and Goldstein, G. Characteristics of preleukemia cells induced in mice. Cell. Immunol., *37:* 308–314, 1978.
21. Hays, E. F. Bone marrow progenitor cells of AKR mice give rise to thymic lymphoma cells. Leukemia Res., *6:* 429–432, 1982.
22. Herzenberg, L. A., and Herzenberg, L. A. Analysis and separation using the fluorescence activated cell sorter (FACS). *In:* D. M. Weir (ed.), Handbook of Experimental Immunology, Ed. 3, Vol. 2, pp. 22.1–22.21. Oxford, England: Blackwell Scientific Publication, 1978.
23. Houben-Defresne, M. P., and Boniver, J. Thymic nurse cells account for the thymus dependency of preleukemic cells in mice after inoculation of radiation leukemia virus. Leukemia Res., in press, 1983.
24. Houben-Defresne, M. P., Varlet, A., Goffinet, G., and Boniver, J. Thymic nurse cells are the first site of virus replication after inoculation of the radiation leukemia virus. Leukemia Res., *6:* 231–241, 1982.
25. Houben-Defresne, M. P., Varlet, A., Goffinet, G., Thiry, A., and Boniver, J. Thymic nurse cells: a site for the interactions between radiation leukemia virus and thymus immature lymphoid cells. *In:* S. J. Baum, G. D. Ledney, and S. Thierfelder (Eds.), Experimental Hematology To-day 1982, pp. 179–186. Basel: S. Karger AG, 1982.
26. Kadish, J. L., and Basch, R. S. Hematopoietic thymocyte precursor. II. A population of thymocytes with the capacity to return ("home") to the thymus. Cell. Immunol., *30:* 12–14, 1977.
27. Kaplan, H. S. The role of cell differentiation as determinant of susceptibility to virus carcinogenesis. Cancer Res., *21:* 981–983, 1961.
28. Kyewski, B., Hunsman, G., Friedrich, R., Ketelsen, U. P., and Wekerle, H. Thymic nurse cells: intraepithelial thymocyte sojourn and its possible relevance for the pathogenesis of AKR lymphomas. *In:* R. Neth, R. Gallo, T. Graf, K. Mannweiler, and K. Winkler (Eds). Modern Trends in Human Leukemia IV, Vol. 26, pp. 372–376. Berlin: Springer Verlag, 1981.
29. Kyewski, B. A., and Kaplan, H. S. Lymphoepithelial interactions in the mouse thymus: phenotypic and kinetic studies on thymic nurse cells. J. Immunol., *128:* 2287–2294, 1982.
30. Kyewski, B., Rouse, R. V., and Kaplan, H. S. Thymocyte rosettes: multicellular complexes of lymphocytes and bone marrow derived stromal cells in the mouse thymus. Proc. Natl. Acad. Sci. U. S. A., *79:* 5646–5650, 1982.
31. Lagerlof, B. A., and Kaplan, H. S. Specificity of the relationship between thymic alkaline phosphatase activity and lymphoma development in strain C57BL mice. J. Natl. Cancer. Inst., *38:* 437–458, 1967.
32. Lieberman, M., Decleave, A., and Kaplan, H. S. Rapid *in vitro* assay for thymotropic, leukemogenic murine C-type viruses. Virology, *90:* 274–278, 1978.
33. Lieberman, M., Decleve, A., Ricciardi-Castagnoli, P., Boniver, J., Finn, O. J., and Kaplan, H. S. Establishment, characterization and virus expression of cell lines derived from radiation- and from virus-induced lymphomas of C57BL/Ka mice. Int. J. Cancer, *24:* 168–177, 1979.
34. Lieberman, M., and Kaplan, H. S. Leukemogenic activity from radiation induced lymphoid tumors of mice. Science Wash. D. C., *130:* 387–388, 1959.
35. Loken, M. R., Stout, R. D., and Herzenberg, L. A. Lymphoid cell analysis and sorting. *In:* M. R. Melaned, P. F. Mullaney, and M. L. Mendelsohn (eds.), Flow cytometry and sorting, pp 505–530. New York Wiley & Sons, Inc., 1979.
36. Mathieson, B. J., Sharrow, S. O., Rosenberg, Y., and Hammerling, U. $Lyt^{1+}23^{-}$ cells appear in the thymus before $Lyt\text{-}123^{+}$ cells. Nature (Lond.), *289:* 179–181, 1981.
37. Molnar, I. The use of rhodizonate in enzymatic histochemistry. Stain Technol., *2:* 5–22, 1952.
38. Reale, E., and Luciano, L. Effect of fixation on the alkaline phosphatase activity of mouse proximal convoluted tubule. J. Histochem. Cytochem., *15:* 413, 1967.
39. Reale, E., and Luciano, L. Kritische elektronenmikroskopische Studien über die Lokalisation der Aktivität alkalischer Phosphatase in Hauptstück der Niere von Maüsen. Histochemie, *8:* 302–314, 1967.
40. Ritter, M. A., Sauvage, C. A., and Cotmore, F. The human thymus microenvironment: *in vivo* identification of thymic nurse cells and other antigenically distinct subpopulations of epithelial cells. Immunology, *44:* 438–446, 1981.
41. Siegler, R., and Rich, M. A. Significance of increased alkaline phosphatase activity in viral induced thymic lymphoma. Proc. Soc. Exp. Biol. Med., *125:*

868–871, 1967.
42. Silverstone, A., Cantor, H., Goldstein, G., and Baltimore, D. Terminal deoxynucleotidyl transferase is found in prothymocytes. J. Exp. Med., *144,* 543–548, 1976.
43. Smith, C. Studies on the thymus of the mammal. XII. Histochemistry of the thymuses of C57BL/6 and AKR strain of mice. J. Natl. Cancer Inst., *26:* 389–403, 1961.
44. Smith, C. Studies on the thymus of the mammal. XIII. Histochemistry of irradiated thymuses of C57BL strain mice. J. Natl. Cancer Inst., *29:* 375–388, 1962.
45. Vakharia, D. D. Demonstration of keratin filaments in thymic nurse cells (TNC) and alloreactivity of TNC-T cell population. Thymus, *5:* 43–52, 1983.
46. Weissman, I. L. Thymus cell migration. J. Exp. Med., *126:* 291–304, 1967.
47. Wekerle, H., and Ketelsen, U. P. Thymic nurse cells-Ia bearing epithelium involved in T-lymphocyte differentiation? Nature (Lond.), *283:* 402–404, 1980.
48. Wekerle, H., Ketelsen, U. P., and Ernst, M. Thymic nurse cells. Lymphoepithelial cell complexes in murine thymuses: morphological and serological characterization. J. Exp. Med., *151:* 925–944, 1980.

Fig. 1. Section of the thymus subcapsular region in a 16- to 18-day-old fetus showing a discontinuous APase-positive reaction on the plasma membrane of lymphoblasts (*Lb*). Uranyl acetate, × 7,200.

Figs. 2 to 4. Thymus of 30-day-old young adult mice. APase activity is observed in plasma cells (Fig. 2), in endothelial cells (Fig. 3, *End*) and in some cortical lymphoid cells associated with epithelial cells (Fig. 3, *arrows*; Fig. 4). Fig. 2, uranyl acetate and lead citrate, × 9,300; Fig. 3, uranyl acetate, × 4,100; Fig. 4, uranyl acetate, × 7,600.

Figs. 5 and 6. APase activity in TNCs. *E*, nucleus of the epithelial cell. Fig. 5, uranyl acetate, × 6,400; Fig. 6, uranyl acetate, × 5,500.

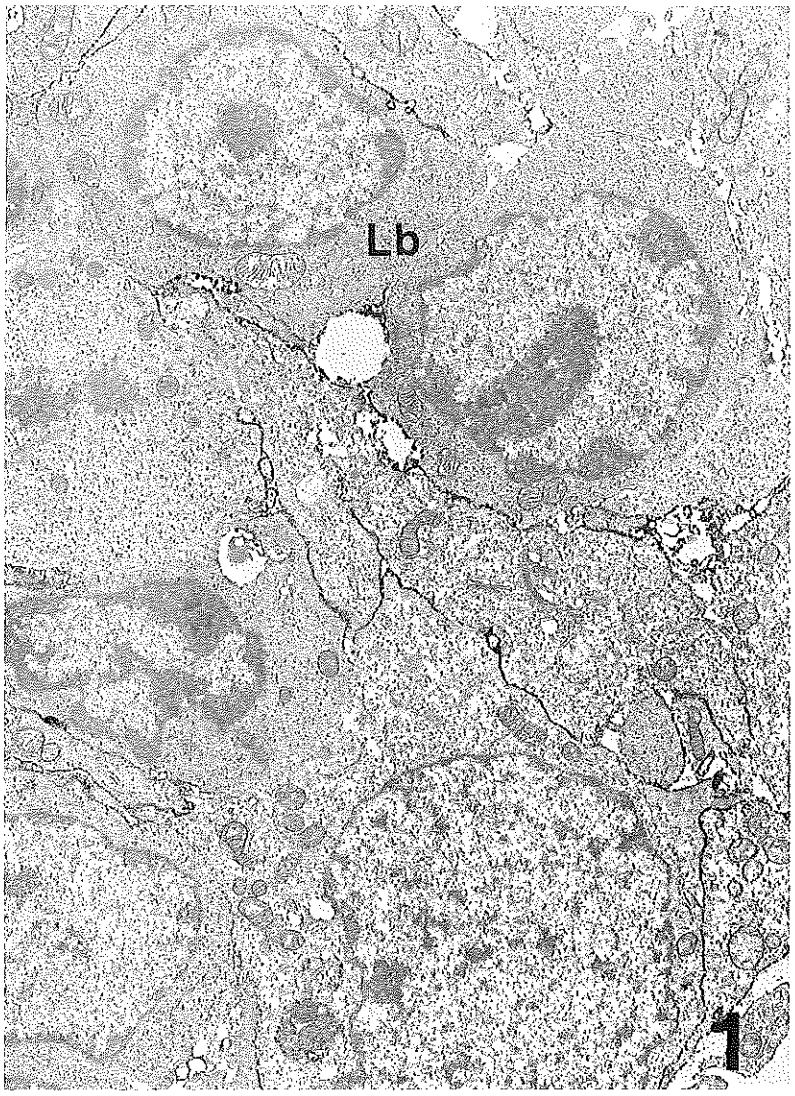
Fig. 7. APase-positive TNC lymphoblast released after mild sonication. *T*, part of a disrupted TNC complex. Uranyl acetate, × 9,000.

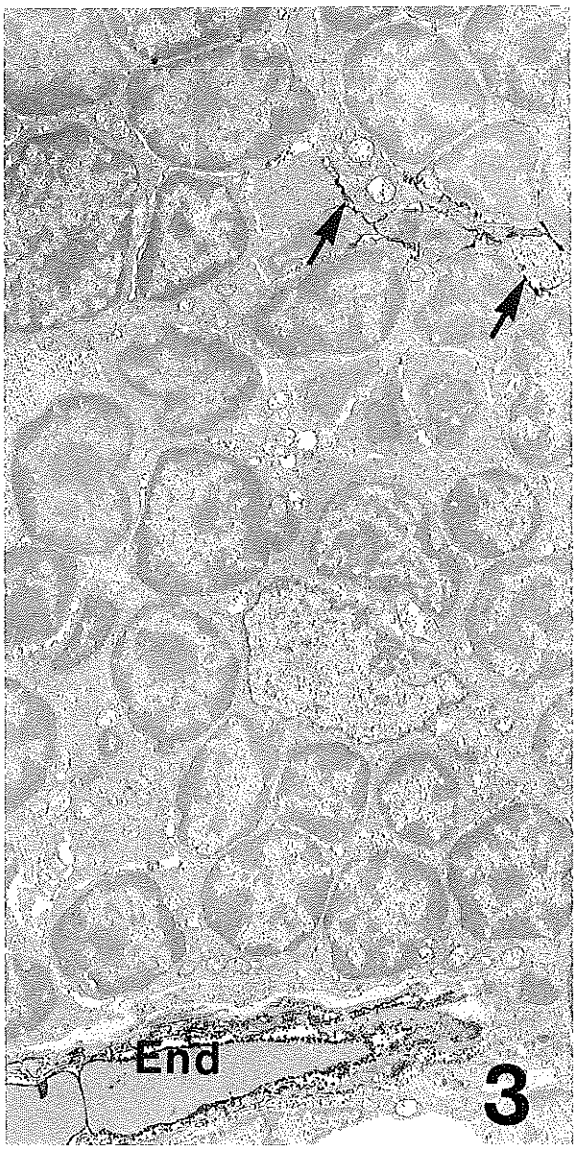
Fig. 8. APase-positive "epithelial cell" in the outer cortex of thymus 2 weeks after inoculation with RadLV. Uranyl adetate, × 10,000.

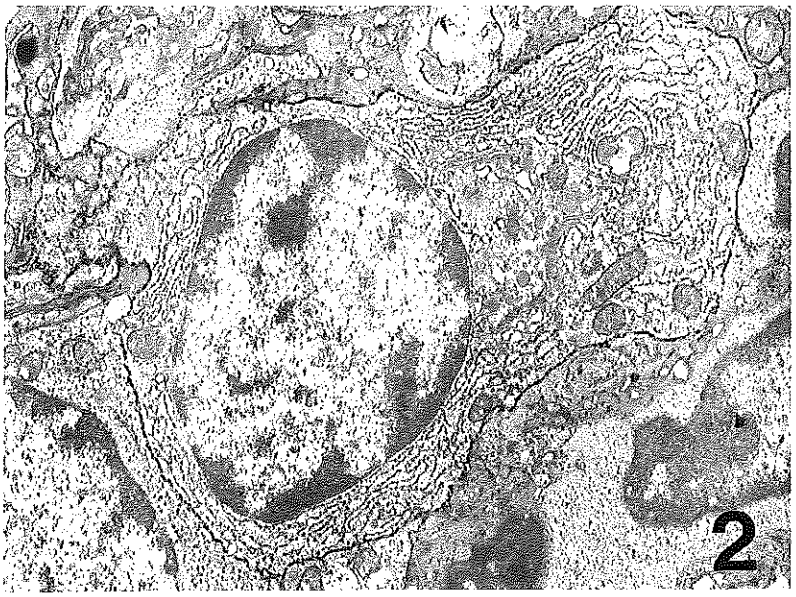
Figs. 9 and 10. Sections of the thymus outer cortex 2 weeks after RadLV inoculation showing membrane APase activity in lymphoblasts (*arrows*). Fig. 9, uranyl acetate, × 10,500; Fig. 10, uranyl acetate, × 8,000.

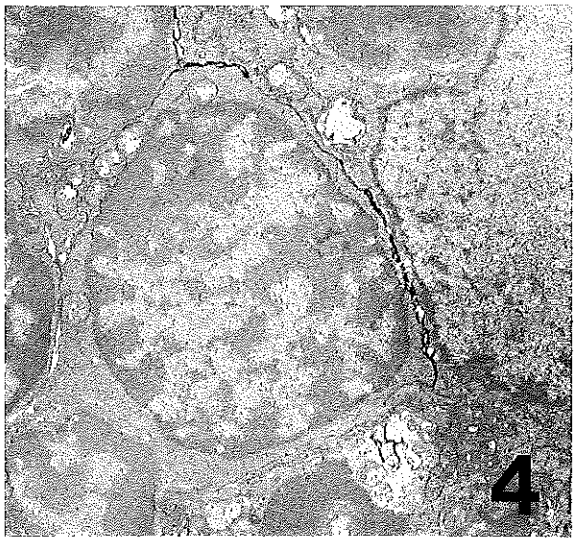
Fig. 11. Area of APase-positive lymphoid blast cells in thymus excised 88 days after RadLV inoculation. Uranyl acetate, × 7,600.

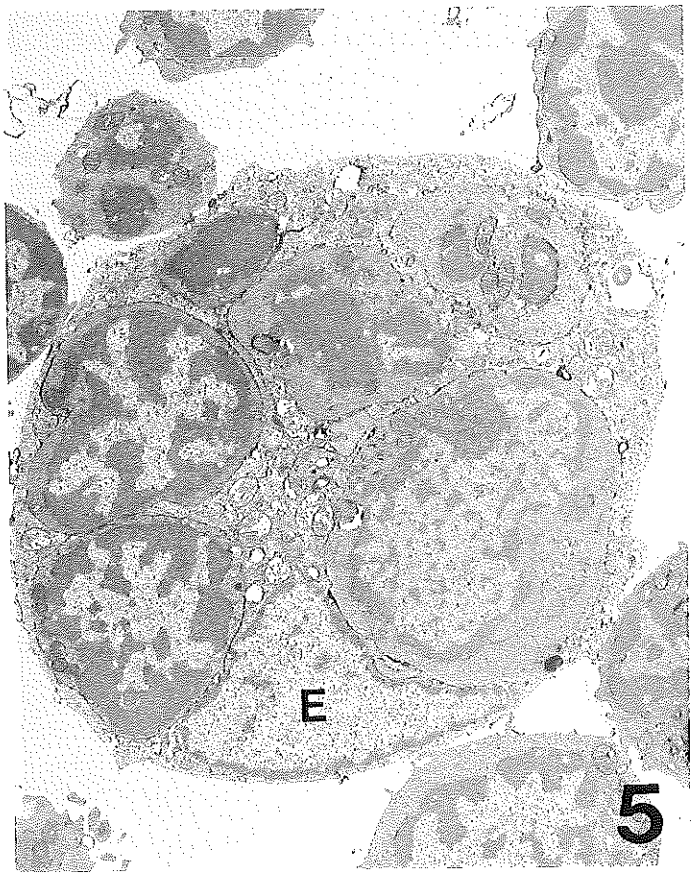
Fig. 12. RadLV-induced lymphoma exhibiting a characteristic extensive APase-positive reaction. Uranyl acetate, × 5,500.

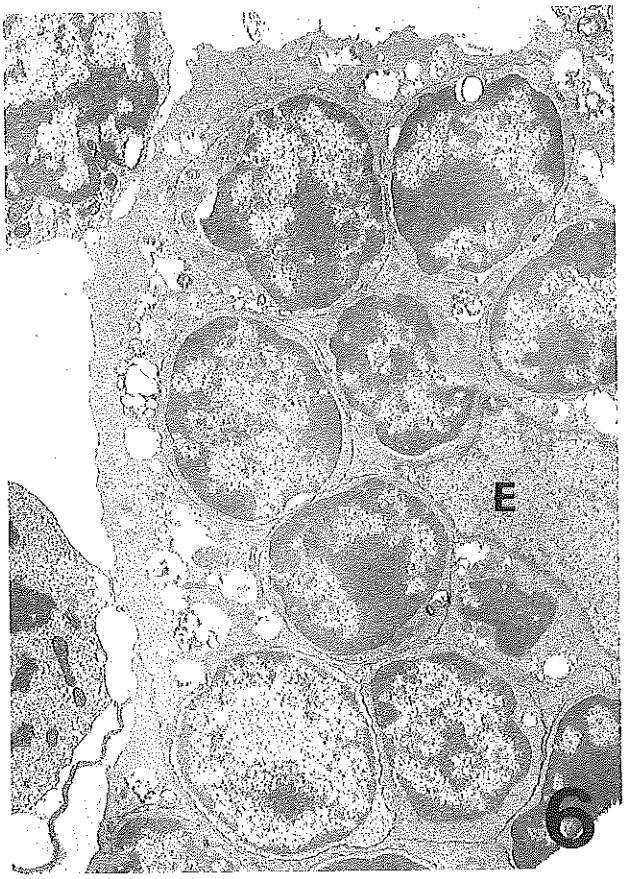

Lb
1

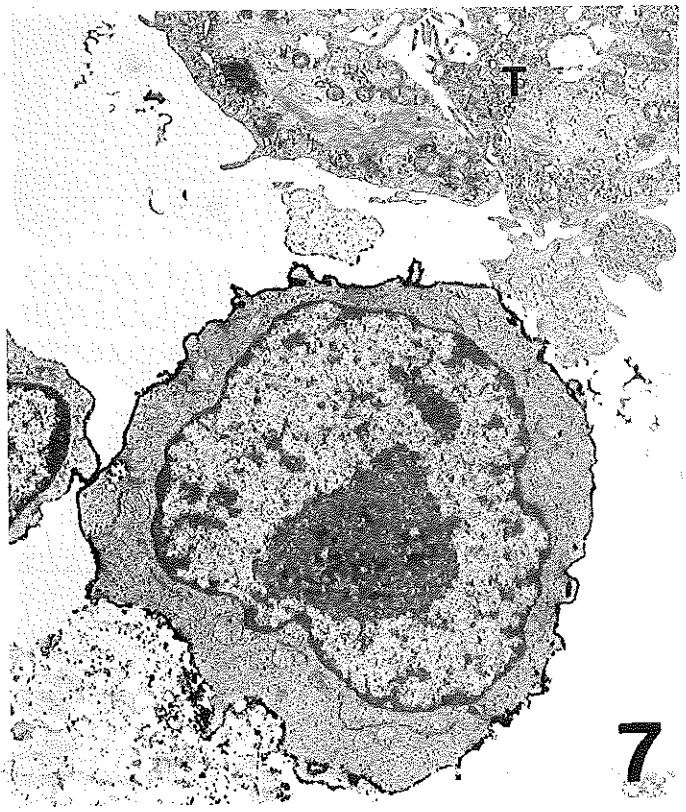

End
3

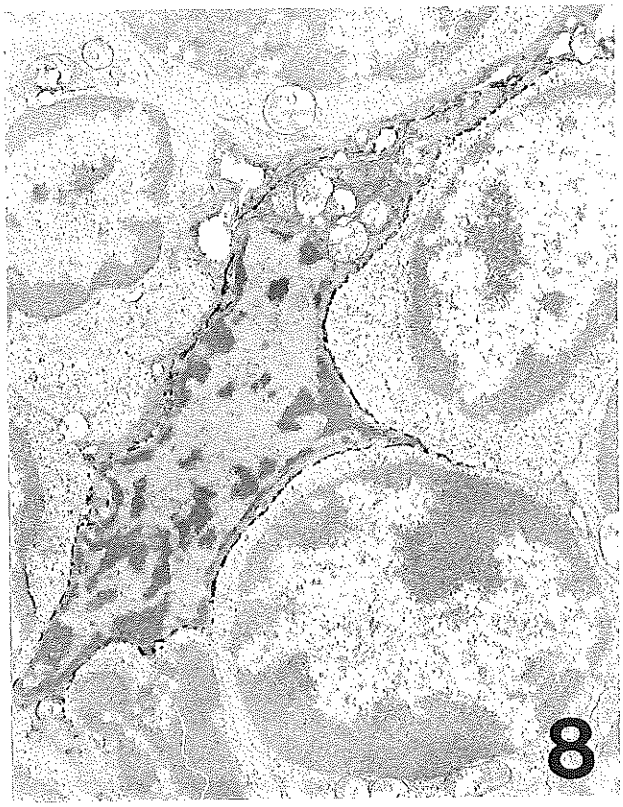

2

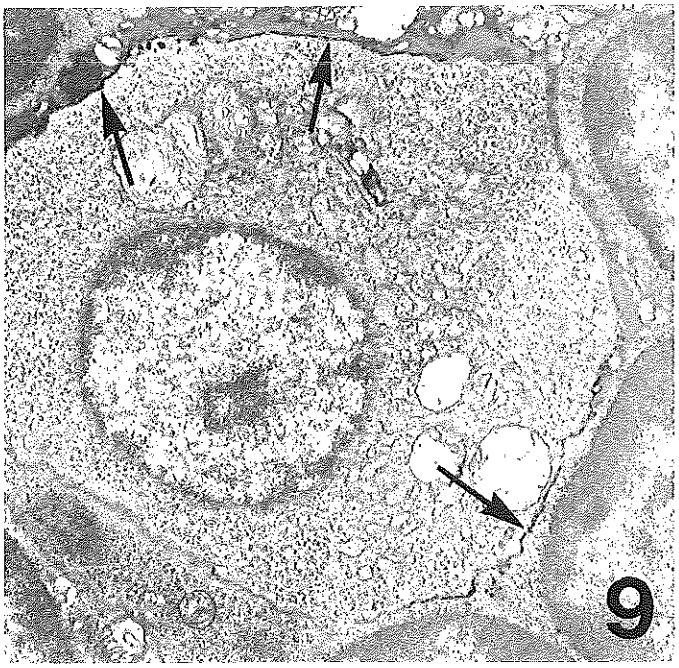

4

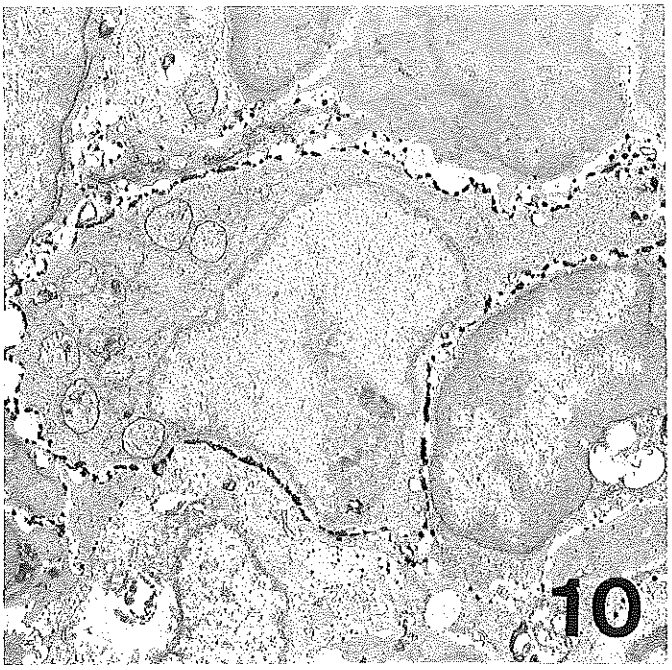

E
5

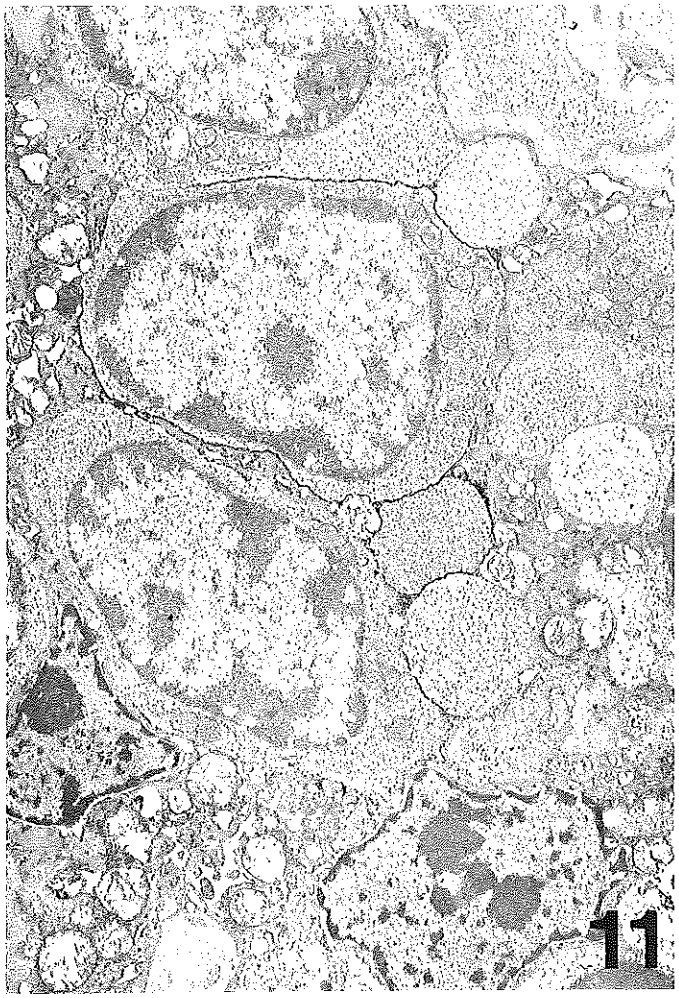

E
6

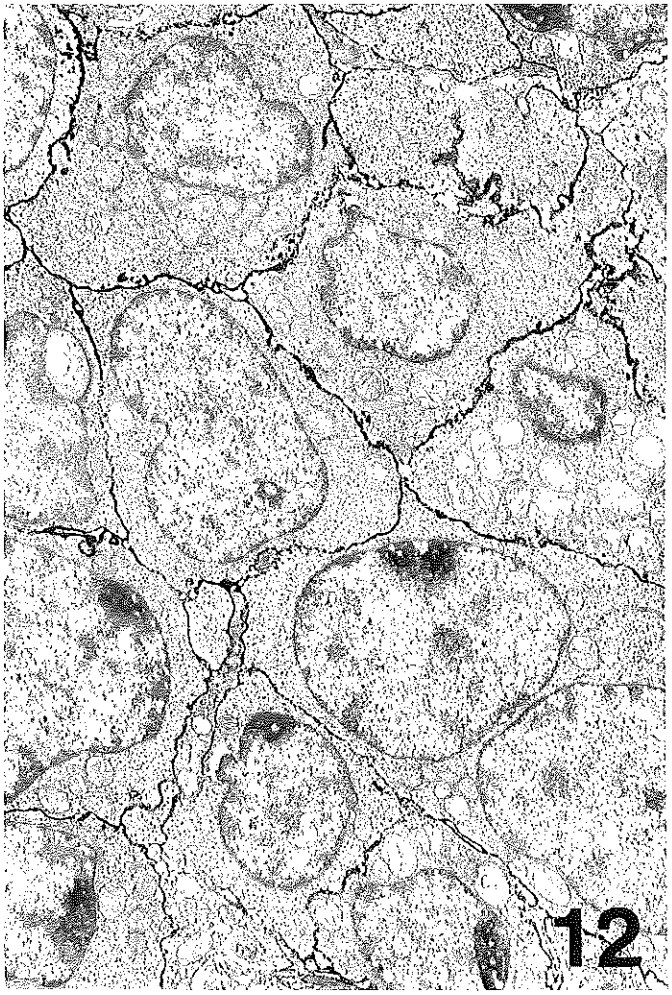

T
7


8


9


10


11


12

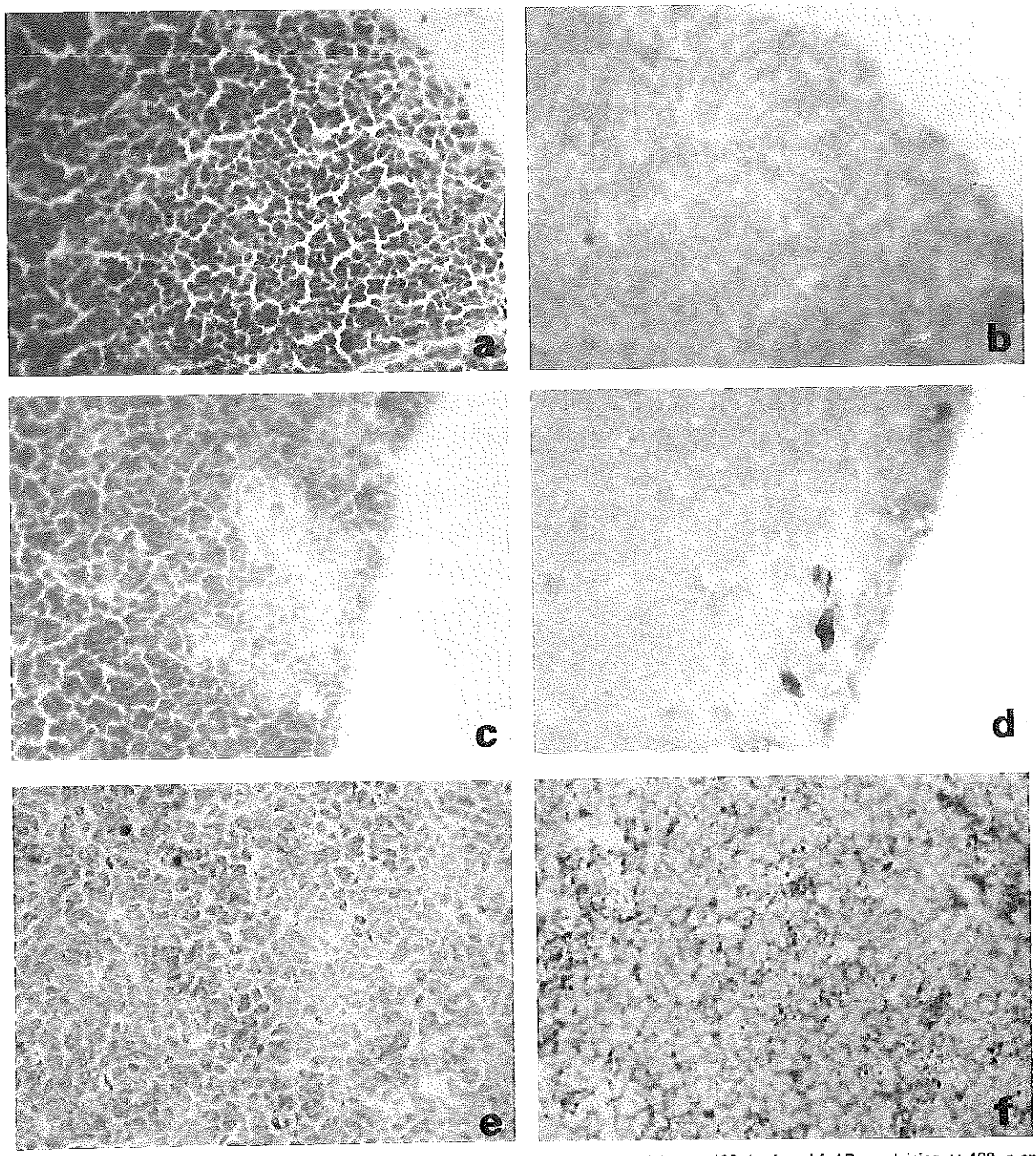


Fig. 13. APase in the thymus after inoculation of RadLV. *a*, *c*, and *e*, pyronine methyl green staining, × 400. *b*, *d*, and *f*, APase staining, × 400. *a* and *b*, thymus cortex after intrathymic inoculation of PBS. The APase expression is not detected at the light microscopy level; *c* and *d*, thymus cortex on Day 90 after intrathymic inoculation with RadLV. Some lymphoid blast cells in the depleted area express APase; *e* and *f*, thymic lymphoma. The majority of tumoral lymphoblasts express APase.