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Alterations of Thymus Cortical Epithelium and Interdigitating Dendritic Cells but No Increase of Thymocyte Cell Death in the Early Course of Simian Immunodeficiency Virus Infection

J.G. Müller,* V. Krenn,† C. Schindler,* S. Czub,* C. Stahl-Hennig,‡ C. Coulibaly,‡ G. Hunsmann,‡ C. Kneitz,§ T. Kerkau,§ A. Rethwilm,‖ V. terMeulen,‖ and H.K. Müller-Hermelink*

From the Institute of Pathology, University of Würzburg; Histology and Embryology Institute,† University of Vienna; German Primate Center,‡ Göttingen; Institute of Immunology,§ University of Würzburg; and Institute of Virology‖ University of Würzburg*

The role of the thymus in the pathogenesis of simian acquired immunodeficiency syndrome was investigated in 18 juvenile rhesus monkeys (Macaca mulatta). The thymus was infected from the first week post-SIVmac inoculation, but the amount of virus-positive cells was very low (< 1 in 10^4 T cells) as demonstrated by polymerase chain reaction and in situ hybridization. First morphological alteration was a narrowing of the cortex at 12 and 24 wpi. Morphometry revealed no increase of pyknotic T cells but a decrease of the proliferation rate and flow cytometry showed a reduction of the immature $CD4^+/CD8^+$ double-positive T cells. Ultrastructural analysis revealed vacuolization, shrinkage, and finally cytolysis of the cortical epithelial cells and the interdigitating dendritic cells. Immunofluorescence staining exhibited a widespread loss of cortical epithelial cells. This damage to the thymic microenvironment could explain the breakdown of the intrathymic T cell proliferation. It preceded fully developed simian acquired immunodeficiency syndrome and is therefore considered to play a major role in its pathogenesis. (Am J Pathol 1993, 143:699–713)

It is generally accepted that the infection by human immunodeficiency virus (HIV) results after several years in an acquired immunodeficiency syndrome (AIDS) in man. However, the mechanisms leading to the immunodeficiency are largely unknown because the results from *in vitro* investigations are not transferable to the more complex situation *in vivo*.[1] *In vitro* infections of susceptible cell lines with HIV result in large scale virus replication and rapid cell death by various direct cytopathogenic effects.[1] *In vivo* only a low number of $CD4^+$ T cells contain proviral HIV-DNA, and most of these infected cells do not replicate the virus.[2,3] This discrepancy prompted the search for indirect mechanisms causing T cell loss *in vivo*. The thymus as the central organ of the T cell development would be an ideal target for HIV infection for several reasons. During the intrathymic T cell maturation, all thymocytes express transiently the CD4 molecule and proliferate rapidly, and it was shown that human thymocytes are infectible by HIV *in vitro*.[4–6] If this would occur *in vivo*, a large scale virus replication within the thymus might result in a destruction of the thymus already in the early course of infection. First results in HIV-infected severe combined immunodeficient (SCID)-hu mice seem to support this hypothesis.[7] The consequences of such a destruction of the thymus would depend upon the age of the patient. In early infancy, destruction of the thymus renders the development of the T cell system impossible.[8] In children and young adults up to the age of about 40 years, the thymus is the prerequisite for a restoration of the peripheral T cell pool as was seen in bone marrow transplantations that are not successful in patients with a severely altered or missing thymus.[9,10]

Postmortem examination of human AIDS thymuses showed a severe atrophy, but in this final stage a dis-

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Address reprint requests to Dr. Justus Müller, Pathologisches Institut der Universität, Josef-Schneider-Str 2, 97080 Würzburg, Germany.

tinction between possible HIV-induced thymus alterations and accidental thymus involution caused by cachexia and/or stress was impossible.[11] Only sporadic investigations of the thymus of HIV-infected man before death could be conducted,[12] and systematic studies are obviously limited to animal models. Among these, the simian immunodeficiency virus(SIV) infection of rhesus monkeys is well-suited for morphological and pathogenetic studies.[13] Both SIV and HIV share the same cell tropism by using the CD4 receptor to infect cells, and there is a 70% nucleotide homology between SIV and HIV-2.[14] SIV-infected rhesus monkeys develop a persistent lymphadenopathy and 1 to 2 years postinfection an immunodeficiency quite comparable to the HIV-induced immunodeficiency in man.[15]

Using this animal model, it was recently reported that the thymus is infected very early and that thymus atrophy precedes the development of the immunodeficiency, but by polymerase chain reaction (PCR) analysis, the authors detected 1 copy of proviral SIV-DNA in only 0.005% of the thymocytes.[16] This is quite low compared with the—estimated—80 to 90% of the daily production of thymocytes being programmed to die naturally,[17] and therefore the question arises how this low amount of SIV causes thymus atrophy. To investigate the pathogenesis of SIV-induced thymus atrophy, juvenile rhesus monkeys with fully developed thymus were infected with SIVmac. The animals were sacrificed at defined time points in the early phase of infection before the development of immunodeficiency or clinical disease so that possible interactions of SIV with the thymus and lymph nodes could be studied unaltered by opportunistic infections or intercurrent diseases. The findings were then compared to animals with end-stage simian AIDS and to the control animals, in the following indicated as controls only.

Material and Methods

Animals and Experimental Design

The 18 juvenile rhesus monkeys (*Macaca mulatta*) were housed individually and tested for SIV, simian T cell lymphotropic virus-1, foamy virus, and type-D retrovirus before the beginning of the experiment.[18] Animals nos. 1 to 12 (Table 1) were infected intravenously with 100 MID_{50} (monkey infectious dose infecting 50% of the recipients) of the *in vivo* titrated cell-free SIVmac251–32H virus stock solution, kindly provided by Dr. M. Cranage (Center for Applied Microbiology and Research, UK).[19] Animal no. 13 was infected intrathecally with 1 ml of SIVmac239 containing 10^3 to 10^4 $TCID_{50}$ (tissue culture infectious dose infecting 50% of the tissue cultures).[20] Animals nos. 14 to 18 served as non-SIV-infected controls.

In the first group imported from China (animals nos. 1 to 8, see Table 1), one animal was sacrificed at 1, 3, 6, 12, 24, 66, and 122 weeks postinfection (wpi) according to the experimental schedule, and animal no. 8 at 124 wpi because of severe clinical disease. In the second group consisting of monkeys of Indian origin (animal no. 9 to 12), the animals had to be sacrificed because of severe clinical disease at 20, 31, 36, and 46 wpi. Animal no. 13 was killed at 31 wpi because of lumbal kyphosis. Using a mixture of ketamine, xylacin, and atropin, the animals were deeply anesthetized and then killed by exsanguination via the femoral vein and the abdominal aorta to obtain the whole blood. All the lymphatic tissues were removed sterilely and then processed immediately for the different experiments.

Table 1. *Animals and Experimental Design*

Source of *M. mulatta*	Chinese								Indian					Chinese		Indian		
Weeks postinfection	1	3	6	12	24	66	122	124	20	31	36	46	31	Controls				
Animal number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Virus inoculum*	32H	32H	32H	32H	32H	32H	32H	32H	32H	32H	32H	32H	239	-	-	-	-	-
Age at death (yr-mo)	3–1	3–1	3–6	3–6	3–10	3–10	4–8	4–3	4–11	2–3	5–3	4–6	2–4	4	18	3–10	8–3	17–9
Gender	f	f	f	m	m	f	f	m	m	m	f	f	f	f	f	m	m	f
Killed according to experimental schedule	x	x	x	x	x	x	x						x1†	x	x	x	x	x
AIDS-defining disease								x	x	x	x	x						

* 32H: 100 MID50 of SIVmac251–32H; 239: 10^3-10^4 TCID of the molecular clone of SIVmac239.
† This animal was killed because of lumbar kyphosis.

Control of SIV Infection, in Vitro *Virus Reisolation, and PCR Analysis*

Following infection, the sera were checked regularly for SIV-specific antibodies by Western blot and p27 enzyme-linked immunosorbent assay (Becton-Dickinson, Mountain View, CA). Virus was reisolated from the peripheral blood and after autopsy from the lymph nodes and the thymus from both cell-free supernatants of homogenized cells and from intact cultured cells by co-culture with the target cell line C8166.[21] Virus antigen was detected in the p27 enzyme-linked immunosorbent assay (Coulter, Luton, UK). PCR analysis of thymocytes was done with a primer recognizing the gag gene segment (AIDS Reagent Project, MRC, UK). Thirty-five cycles were run using taq-polymerase (Promega, Heidelberg, Germany), and the amplified DNA was separated using gel electrophoresis and visualized by ethidium bromide staining. The minimal amount of thymocytes creating a positive signal was determined by limited dilution (C. Kneitz, unpublished).

Routine Histology

The architecture of the thymus, including the size of cortex and medulla, the cortico-medullary stratification, the content of Hassalls corpuscles, and the size of the perivascular space, was evaluated in 1-μ paraffin sections stained with hematoxylin and eosin, Giemsa, periodic acid-Schiff, and Gordon Sweet. The lymph nodes were taken from the following regions: cervical, axillary, mediastinal, upper intestinal, mesenterial, mesocolic, paraaortal, inguinal, and popliteal. A semiquantitative analysis for follicular lymphatic hyperplasia, alterations of the germinal center, and atrophy of the paracortex was done. Alterations of the germinal centers included loss of the follicular mantle zone, follicular fragmentation, follicular hyalinization, and follicular atrophy.[22] All other organs were investigated for intercurrent diseases.

Immunohistochemistry and Flow Cytometry

One cross-section of the thymus and one-half of a lymph node of each region were snap-frozen in liquid nitrogen and stained with the alkaline phosphatase antialkaline phosphatase (APAAP) technique following standard protocols. Monoclonal antibodies recognizing *Macaca mulatta* T cells (FN 18 against CD3, Margaret Jonker, Amsterdam; Okt 4 and Okt 8, Becton-Dickinson; and MT 122 against CD8, P. Rieker, Munich), B cells (L 26 against CD20, Dako, Copenhagen, Denmark), macrophages (Ki M 8, Kiel, Germany), follicular dendritic cells (Ki M 4p, Kiel, Germany) and proliferating cells (Ki 67, Kiel, Germany) were used. Thymus epithelial cells were stained using 35βH11 (Enzo, recognizing cytokeratins 8 and 18) as the primary and a fluorescein isothiocyanate- (FITC) or tetrarhodamine isothyocyanate (TRITC) conjugated as the secondary antibody. For the detection of SIV proteins, four monoclonal antibodies produced by K. Kent[23] and kindly provided by the AIDS Reagent Project were used: KK8 directed against SIVenv gp120, KK13 against SIV gp160/120 V2-loop, KK20 against SIV gp160/41 aa595–617, and KK 59 against SIV p17. Cells of the thymus reacting with the SIV antibodies were double-stained with CD4, CD8, and Ki M8 using indirect labeling with FITC as the first antigen, followed by incubation with mouse immunoglobulin G and indirect labeling of the second antigen with TRITC. Controls consisted of uninfected monkey tissues and of staining with an unrelated primary antibody.

In double-staining flow cytometry analysis of thymocytes, indirect staining of the first antigen using phycoerythrin was followed by an incubation step with mouse immunoglobulin G and then by direct staining of the second antigen with FITC-conjugated FN18. Appropriate negative controls were used to set the cut-off and to calculate the fraction of positive cells using fluorescence-activated cell sorter (FACS)can research software.[21]

Electron Microscopy

Two cross-sections of the thymus were cut into 1-mm cubes, fixed in 2.5% glutaraldehyde, post-fixed in OsO_4, and embedded in Epon 812. From at least two representative Epon blocks of the thymus, ultrathin sections were prepared on 100 μ × 100 μ meshs, contrasted with $PbCO_3$ and evaluated on a Zeiss EM 10 or EM 109 transmission electron microscope. Photographs were taken from each square of the ultrathin section at 3,200× enlarged to 18 cm × 24 cm prints, and reassembled to a large photocollage. This represented the semithin section on an ultrastructural level and allowed an exact localization and identification of the respective cells.

Morphometry

Semithin sections (0.5-μ thick) from the glutaraldehyde-embedded blocks of the thymus

were stained with toluidine blue. In representative cortical areas from at least five different Epon blocks per animal, the number of mitotic figures and pyknotic cells was counted in consecutive high-power fields (100× oil-immersion objective). The total number of thymocytes in these high-power fields was calculated by counting all thymocytes in six of 36 fields of a square lattice within the ocular. From at least 10,000 cells per animal, the rate of mitotic figures and the rate of pyknotic cells were determined. Statistical correlations were tested using the linear regression method, and statistical differences with a *t*-test using the CSS Statistica software package.

In Situ *Hybridization*

The p5′ SIV-vector was a friendly gift of Dr. R. Desrosiers (Southborough, MA). It was cut with AccI resulting in a 527-bp probe of the gag sequence that was inserted into the vector pGEM4 (Promega). For transcription, the T7 or the SP6 polymerase (Boehringer, Mannheim, Germany) were used in addition of [^{35}S]αUTP (New England Nuclear). Five-μ frozen sections were fixed in acetone and postfixed in 5% paraformaldehyde. Anti-sense and sense probes hybridized overnight at 45 C. The slides were washed twice at 60 C with 2× standard saline citrate puffer. The slides were coated with Ilford K4 solution and exposed for 8 and 16 days at 4 C. Positive controls consisted of tissues of animal no. 9 exhibiting numerous virus particles by electron microscopy and of *in vitro* SIV-infected C8166 cells. All positive controls gave a strong signal with the anti-sense probe but no signal using the sense probe.

Results

Before SIV infection, the animals were seronegative for SIV, D-type retrovirus, and human T cell lymphotropic virus-1, but about 80% were seropositive for foamy virus. Foamy virus is not known to induce disease in macaques.[24] All SIV-infected animals seroconverted at 4 wpi, and virus was isolated from the peripheral blood from 2 wpi until termination of the experiments.[21] Most of the Chinese monkey thymuses, but only few of the Indian monkey thymuses could be studied unaltered by AIDS-defining diseases.

SIV within the Thymus

The thymus was infected from the first week postinfection. This was shown by co-culture of interleukin-2-stimulated thymocytes with the C8166 cell line exhibiting cytopathogenic effects and by a positive p27 enzyme-linked immunosorbent assay (Table 2). By DNA-PCR with SIV-specific primers, a positive signal was generated with at least 10^4 thymocytes (Table 2). Co-culture of the supernatant of homogenized thymus suspensions with the C8166 cell line failed to induce cytopathogenic effects but gave positive results when the supernatant of lymph node homogenates was used. *In situ* hybridization detected 1 to 5 positive cells per slide, each containing about 10^4 to 10^6 cells (Figure 1A). No free viral particles were found by electron microscopy in the thymus, but at 6 wpi (animal no. 2) one budding virus particle was detected in the perivascular space.

Immunohistochemistry with the four monoclonal SIV antibodies (KK8, KK13, KK20, and KK59) revealed only single positive cells in the subcapsular cortex (Figure 1B) with the exception of animal no. 8 (Table 2). In this animal with the most atrophic thymus, the density of positive cells was slightly increased. High numbers of positive cells were detected in the positive controls (SIV-infected C8166 cells, and lymph nodes or brain of monkey no. 9 with SIV encephalopathy). In addition, all four antibodies detected a few positive cells in the thymus

Table 2. *Detection of SIV in the Thymus of the Chinese Animals in the Time Course of SIVmac251–32H Infection*

Weeks postinfection Animal number	Control 14 +16	1 1	3 2	6 3	12 4	24 5	66 6	122 7	124 8
Immunohistochemistry	+	+	+	+	+	+	+	+	++
Electron microscopy	–	–	*	–	–	–	–	–	–
In situ hybridization	–	+	+	+	+	+	+	+	+
Coculture with C 8166									
Homogenized cells	–	–	–	–	–	–	–	–	–
Intact cells	–	pos	pos	pos	pos	pos	pos	pos	pos
PCR	–	about one copy of SIV-DNA in 10^4 thymocytes							

–, no positive signal; +, single cells with positive signal per section; *, one budding virus within the perivascular space; pos, occurrence of syncytial giant cells and positive SIV p27 ELISA.

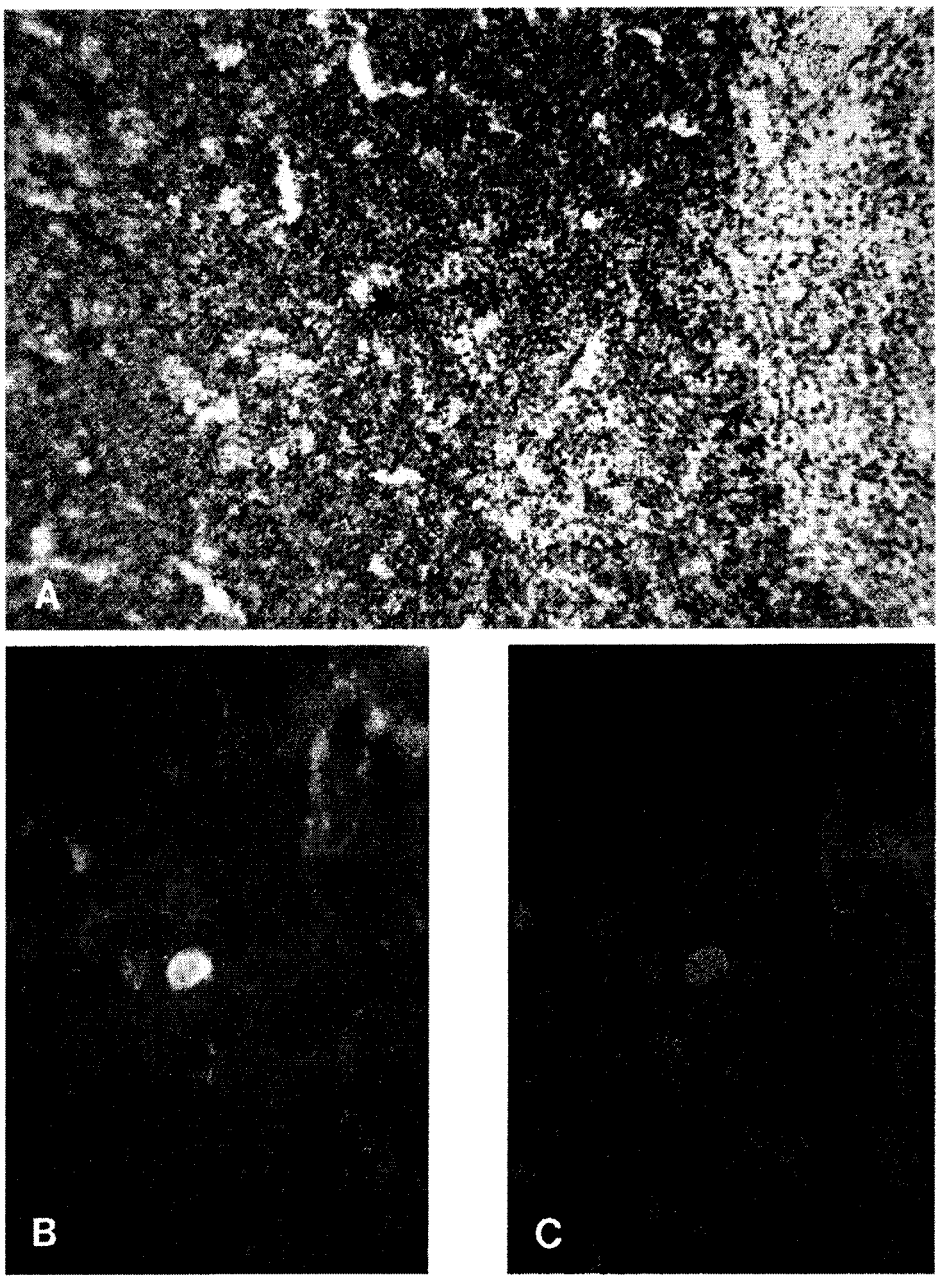

Figure 1. *Demonstration of SIV within the thymus.* **A:** *One of the positive signals as demonstrated by* in situ *hybridization in the thymus cortex of animal no. 2 (3 wpi).* **B** *and* **C:** *Double labeling immunofluorescence demonstrating within a Ki M 8-TRITC-positive macrophage (red) a positive signal for SIVenv as demonstrated by KK8 FITC-immunofluorescence (green) in the thymus cortex of animal no. 2 (3 wpi).*

cortex of non-SIV-infected control monkeys. Double labeling immunofluorescence was positive for macrophages (Ki M 8, Figure 1C), but not for T cells (FN18) or epithelial cells (35βH11).

The Fate of the Thymocytes

The first alteration was a narrowing of the thymus cortex by one-half as compared with the controls (Figure 2A and Table 3). This cortical shrinkage was focal at 12 wpi, already involved the whole cortex at 24 wpi (Figure 2B) and progressed to a total loss of some thymic lobules at 66 wpi (Figure 2C). Acomplete thymus atrophy (Figure 2D) like that in autopsy of human AIDS patients occurred only in animals with fully developed simian AIDS and severe opportunistic infections.

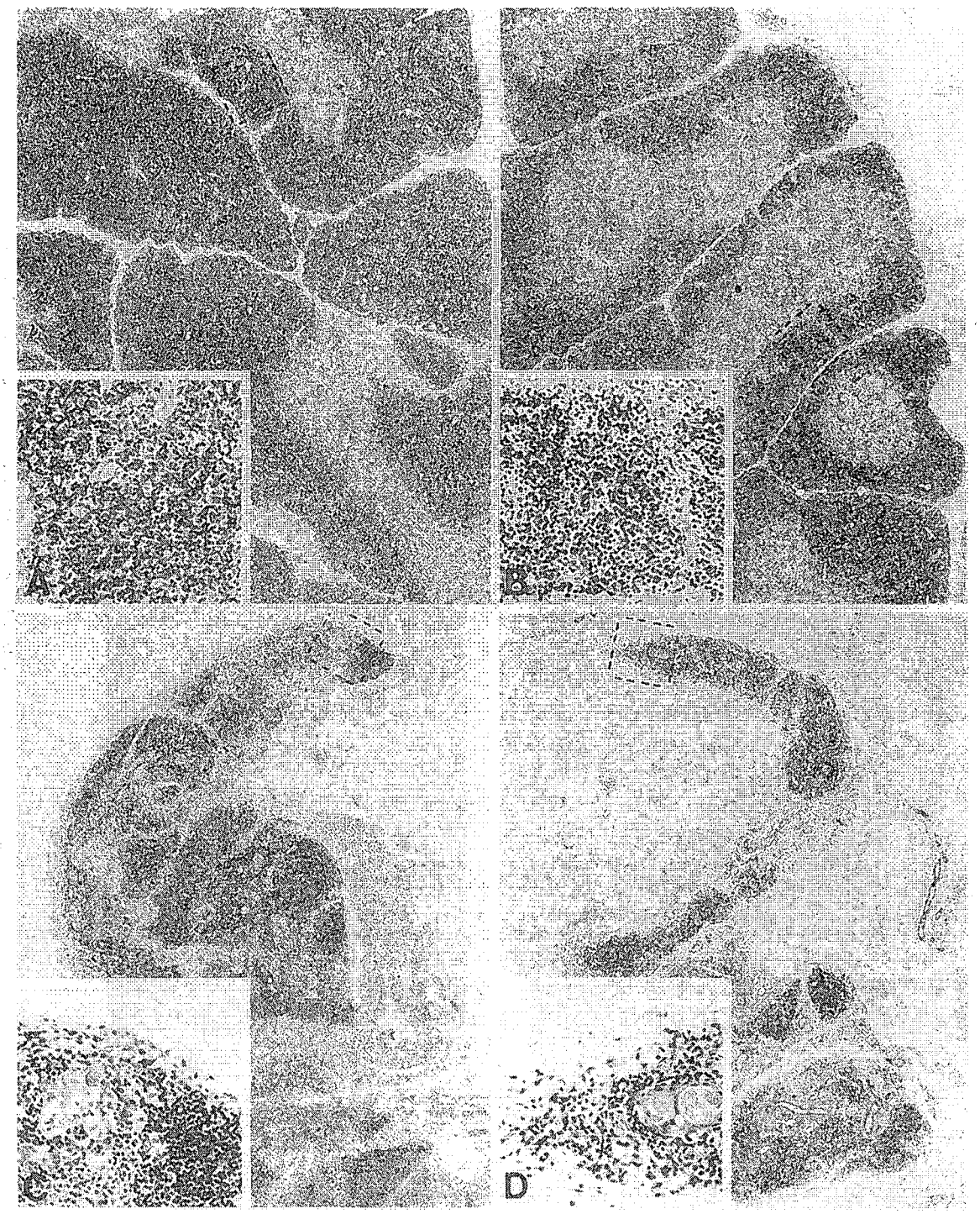

Figure 2. *The size of the thymus in the time course of SIV-infection.* A: *Control, exhibiting a broad cortex.* B: *At 24 wpi, the cortex was narrowed by one-half. Inset: Increased density of small-sized thymocytes in comparison with inset of* A:. C: *At 66 wpi, some lobules were lost and the remaining exhibited a shrinkage of cortex and medulla. Inset: The cortex contains small-sized thymocytes only.* D: *At 124 wpi, with fully developed AIDS, there was complete thymus atrophy. Inset: Some Hassalls corpuscles remained. 28×. Insets 172×. Paraffin, HGE.*

The evaluation of the semithin sections demonstrated in the narrowed cortices a decrease in the number of the lymphoblasts and in correspondence an increase in the small-sized thymocytes (Figure 3, A to D). This parallels the flow cytometry results of a reduction of the double posi-

Table 3. *Alterations of the Thymus of the Chinese Animals in the Time Course of SIVmac251–32H Infection*

Weeks postinfection	controls	1	3	6	12	24	66	122	124
Animal number	14 +16	1	2	3	4	5	6	7	8
Size of the thymus									
Atrophy of cortex	–	–	–	–	+	+	++	++	+++
Atrophy of medulla	–	–	–	–	–	–	+	+	++
T cell subsets of the thymus determined by flow cytometry (% of total)									
DP: $CD3^+4^+8^+$	82			76	69	62			
SP: $CD3^+4^+8^-$	9			8	13	17			
SP: $CD3^+4^-8^+$	5			8	10	10			
TN: $CD3^-4^-8^-$	4			8	8	11			

–, none (compare with Fig. 1A); +, narrowing by one half (compare with Fig. 1B); ++, narrowing by more than one half (compare with Fig. 1C); +++, total loss (compare with Fig. 1D); DP: double positives; SP: single positives; TN: triple negatives.

tive (CD3+/CD4+/CD8+) thymocytes from 82% in the control to 69% at 12 wpi and to 62% at 24 wpi (Table 3). The proportion of CD4+ single-positive thymocytes increased from 9% in the control to 17% at 24 wpi, and the respective proportions of CD3+/CD4–/CD8+ increased from 5% to 10% at 24 wpi.[21] Morphometry revealed a statistically significant ($P < 0.001$) reduction of the mitotic rate from 1.1% in the controls to 0.4% in all narrowed cortices (Figure 4). The same was seen using Ki 67 immunohistochemistry showing patches of nonproliferating cells in the narrowed cortices.

The rate of pyknotic cortical cells was determined as a possible indicator of direct SIV-induced cytopathogenic effects. Instead of an expected increase, we found a decrease from 1.5% in the controls to 1.1% at 1, 3, 6, and 12 wpi, and to 0.5% in all animals with narrowed cortex (Figure 4). Syncytial giant cells are another sign of SIV-induced cytopathogenic effects *in vitro*, but can occur *in vivo* in a variety of other circumstances. In the thymus, seven giant cells were found in animal no. 3, one in animal no. 4, and one in the atrophic AIDS thymus of animal no. 12. Six of them were found in Epon blocks and could be further investigated by electron microscopy (Figure 5A). Only one viruslike particle was found in a cytoplasmic vacuole of the giant cell of animal no. 12. This is in contrast to the giant cells of the brain and the lung of animal no. 9 with SIV encephalopathy. All giant cells of this animal were surrounded by numerous viral particles and in addition contained virus particles within cytoplasmic vacuoles (Figure 5B) and thus resembled the findings in SIV-infected susceptible cell lines.

The Fate of the Cells of the Thymus Microenvironment

The cortical and the subcapsular epithelial cells exhibited a loss of the cytoplasmic processes in all animals with a narrowed cortex, but not in the controls and the animals at 1, 3, and 6 wpi (Figure 6, A, B and C). In addition, these cells showed a wrinkling of the nuclear membrane and increased amounts of secondary lysosomes. These alterations were focal at 24 wpi and generalized at later time points, when cytolysis of the subcapsular and cortical epithelial cells was another frequent finding (Figure 6D). At the light microscopic level, this necrosis of epithelial cells resulted in a loss of the meshwork of the cortical epithelium that was shown by cytokeratin immunofluorescence (Figure 7, A and B). In addition, large cytoplasmic vacuoles were observed at 12 wpi and at later time points in the subcapsular and cortical epithelial cells (Figure 6B). Occasionally, a fine granular material was found within these vacuoles. The interdigitating dendritic cells laying in the cortico-medullary boundary showed a severe shrinkage of the cytoplasm and the nuclei with a loss of the cellular organelles and the cytoplasmic processes (Figure 8, A and B). No ultrastructural alterations of medullary epithelial cells or Hassalls corpuscles were found until now. Mature plasma cells with a well-developed rough endoplasmic reticulum occurred frequently, close to the necrotic cells of both the cortex and the medulla (Figures 6D and 8B).

The perivascular space was enlarged focally by a few small germinal centers containing follicular dendritic cells (KiM4p+) in animals nos. 3 and 4. In atrophic AIDS thymuses, the blood vessels of the perivascular space were densely aggregated, most probably because of a loss of the intervening thymic tissues (Figure 2D). Additional findings were small areas with myelopoiesis within the subcapsular cortex of both controls and some SIV-infected animals.

Lymph Nodes and Other Organs

Beginning at 3 wpi, there was a massive enlargement of the lymph nodes by a follicular lymphatic

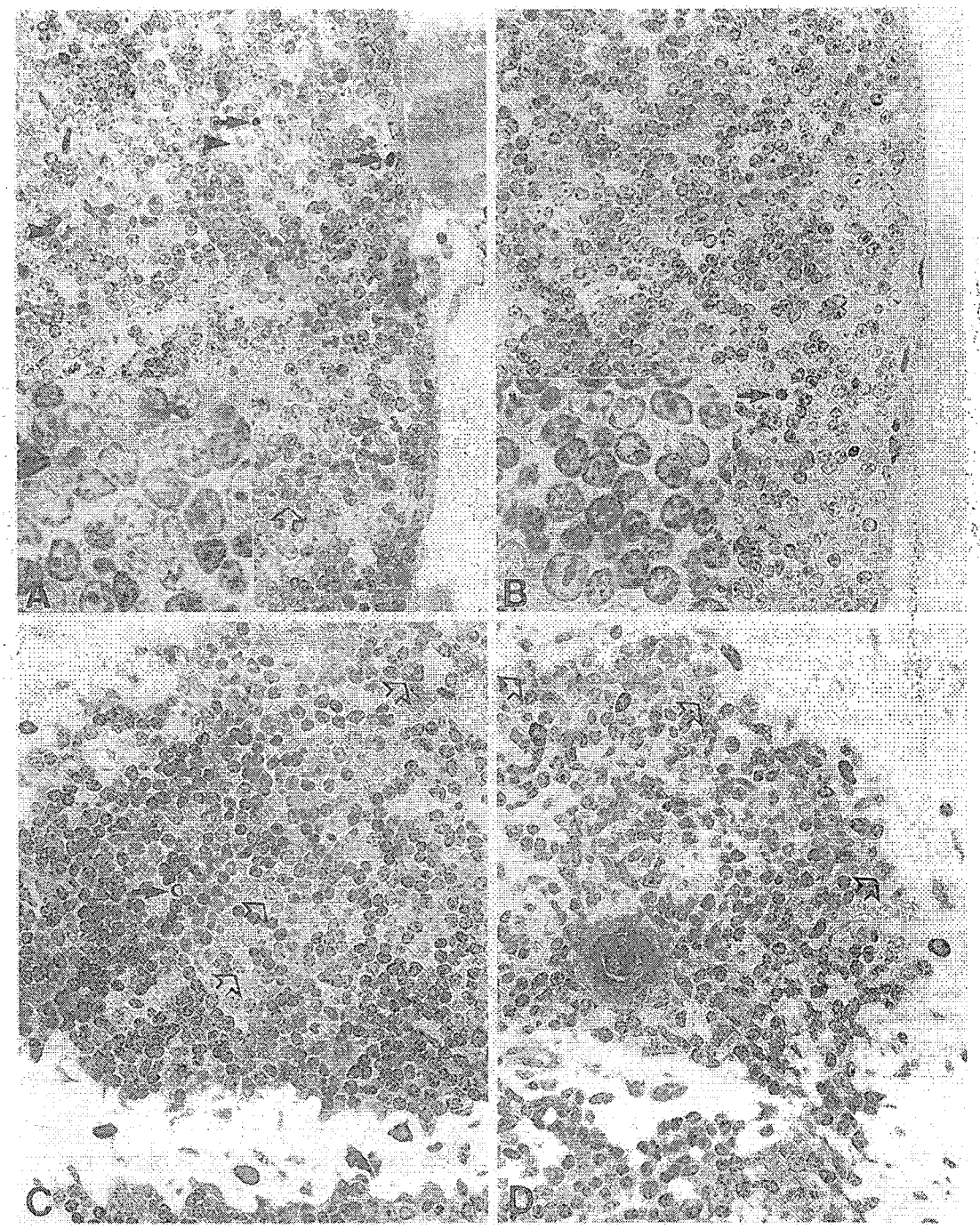

Figure 3. *Cytology of the thymus cortex in the time course of SIV-infection.* **A:** *Control with numerous mitotic figures (arrowheads), pyknotic thymocytes (arrows), and two plasma cells (open arrow). Inset: –blasts with large euchromatic nuclei outnumber –cytes with smaller, more heterochromatic nuclei.* **B:** *At 24 wpi: no mitotic figure, few pyknotic cells (arrows), vacuolization of the subcapsular epithelial cells. Inset: Inversed ratio of –blasts and –cytes.* **C:** *At 66 wpi, folded capsule with mast cells. Cortex with small sized thymocytes, few pyknotic cells (arrow), and some plasma cells (open arrow)* **D:** *At 124 wpi, total loss of the cortex, 1 Hassall corpuscle, numerous plasma cells (open arrows). 345×, insets 819×. Epon, semithin sections, toluidine blue.*

hyperplasia (Table 4). First alterations of the germinal centers occurred at 24 and 66 wpi. At 122 and 124 wpi, most follicles showed atrophy or hyalinization. Atrophy of the paracortex occurred only very late in animal no. 8, suffering from fully developed AIDS and opportunistic infections (Table 4).

The controls and the animals nos. 1 to 5 were free of any intercurrent disease (Table 1). Animal no.

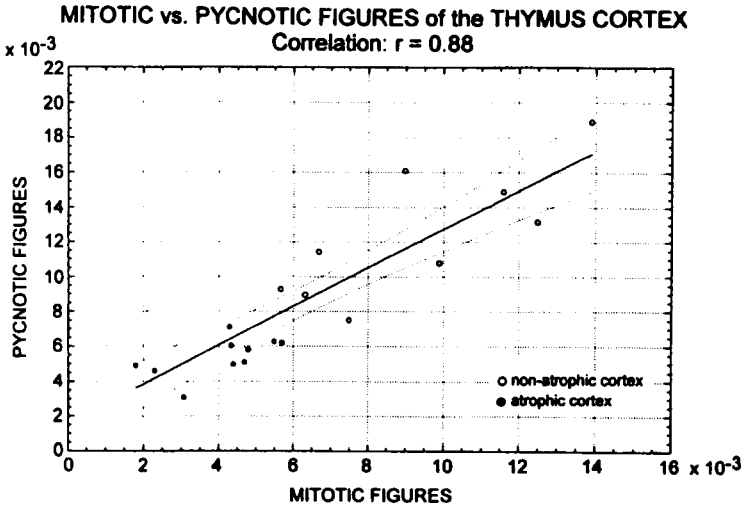

Figure 4. *Morphometry of the thymus cortex. Highly significant statistical association between the rate of mitotic and pyknotic cells in animals with nonatrophic (open circles) and atrophic (closed circles) thymus cortex as demonstrated by linear regression analysis ($P < 0.001$, $r = 0.88$).*

6 was sacrificed at 66 wpi because of weight loss and showed slight nonspecific enterocolitis as well as a small (0.2 cm) extranodal B-cell lymphoma in one adrenal gland. The clinically healthy animal no. 7 was killed at 122 wpi because of termination of the experiment. Animal no. 8 suffered at 124 wpi from AIDS exhibiting cytomegalovirus pneumonia, microfilaria enterocolitis, and atrophy of the lymph nodes and the thymus (Figure 2D).

Most of the Indian animals had to be euthanized in the early time course of SIV infection because of severe clinical diseases (Table 1). Animal no. 9 suffered from SIV encephalopathy, giant cell pneumonia, and generalized lymphoproliferation with focal necrosis involving also the thymus medulla. Animal no. 10 showed a large extranodal B-cell lymphoma of the orbita and a well-developed thymus. Animal no. 11 exhibited a severe vasculopathy of the lungs

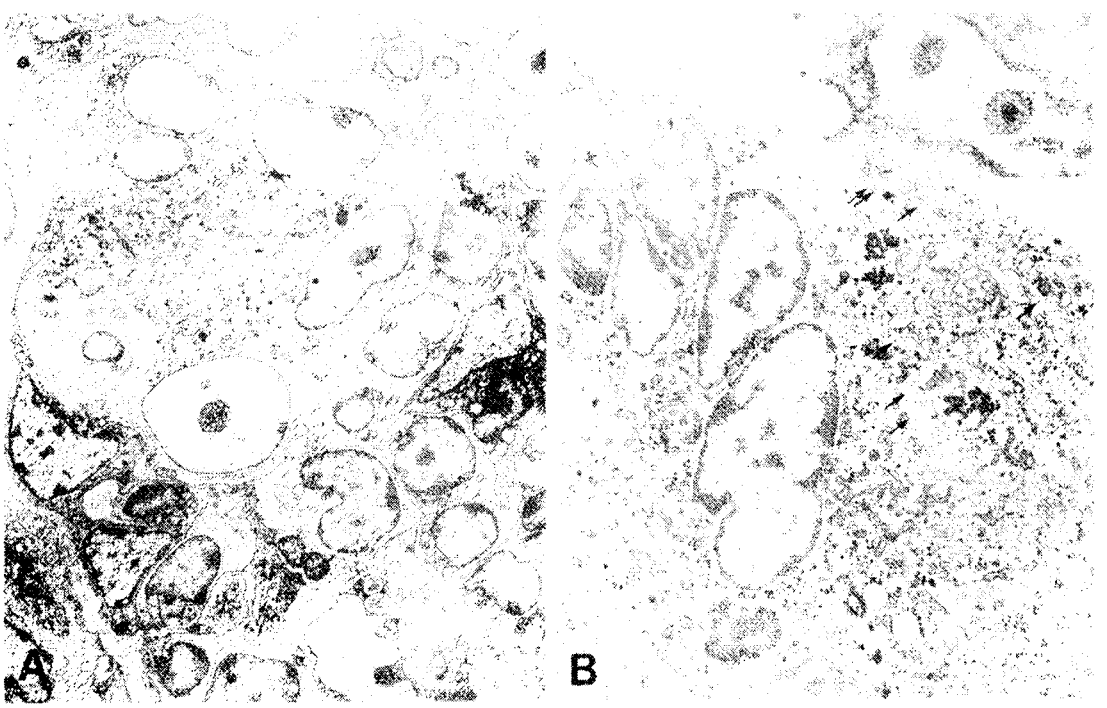

Figure 5. *Giant cells of the thymus and lung in SIV-infection.* A: *Giant cell in the thymus at 6 wpi (2,700×).* B: *Giant cell in the lung of animal no. 9. In this giant cell, numerous viral particles were detected within cytoplasmic vacuoles (arrows and inset). 2,700×, inset 44,000×.*

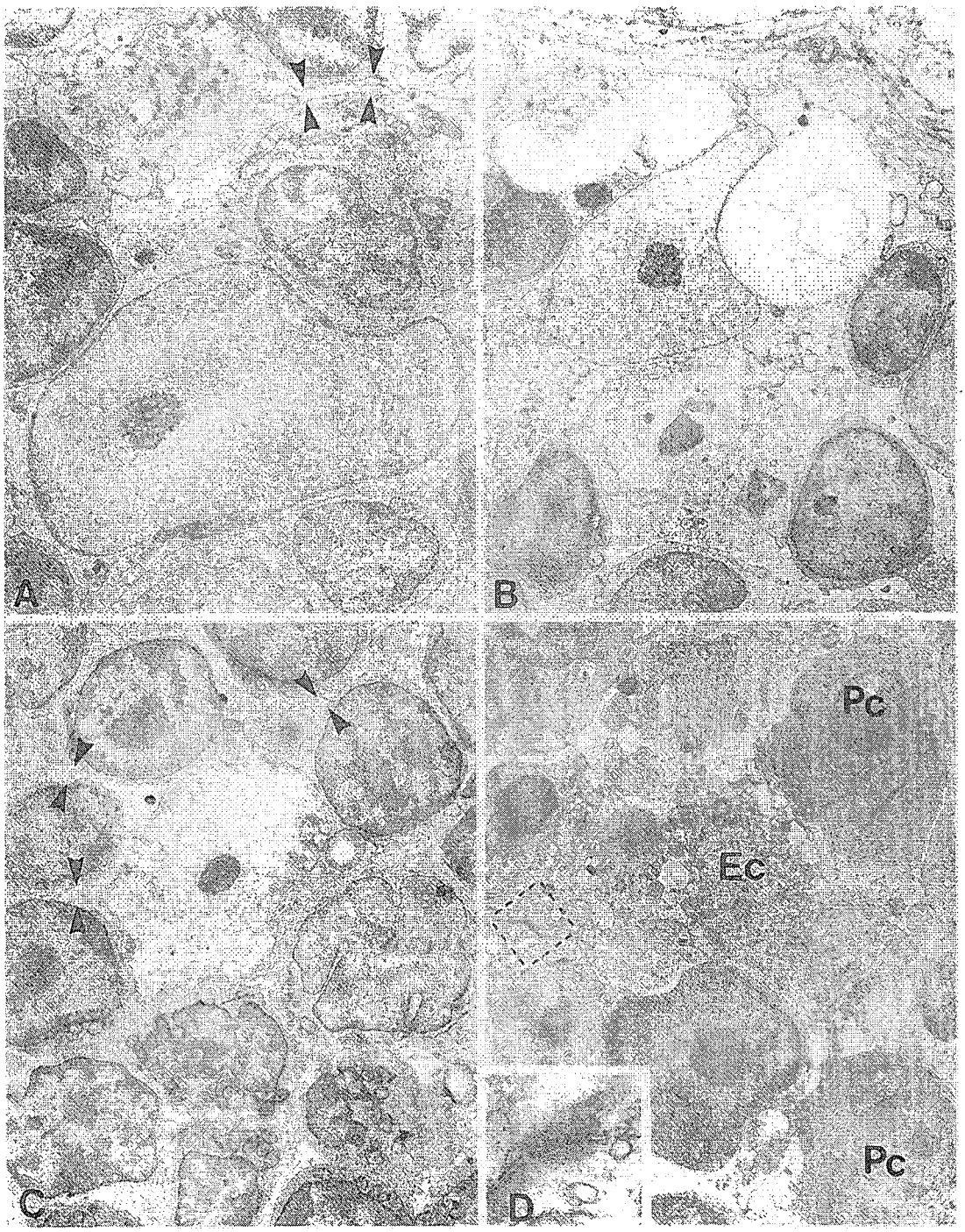

Figure 6. *Ultrastructure of epithelial cells of the cortex of the thymus.* **A:** *Cortical epithelial cell of a control, exhibiting long cytoplasmic processes (arrowheads), which surround adjacent lymphoblasts (6,000×).* **B:** *Vacuolization of a subcapsular epithelial cell at 24 wpi (3,500×).* **C:** *Cortical epithelial cell at 66 wpi exhibiting loss of cytoplasmic processes (arrowheads) and wrinkling of the nuclear membrane (3,500×).* **D:** *Cytolysis of a subcapsular epithelial cell (Ec). Two plasma cells (Pc). Inset: Desmosom. 4,800×, inset 19,200×.*

and the other organs as well as a partial thymus atrophy comparable with Figure 2C. Animal no. 12 had an extranodal B-cell lymphoma of the lungs and upper mediastinum so that no residuals of the thymus were found. The otherwise healthy animal no. 13 was sacrificed because of lumbal kyphosis

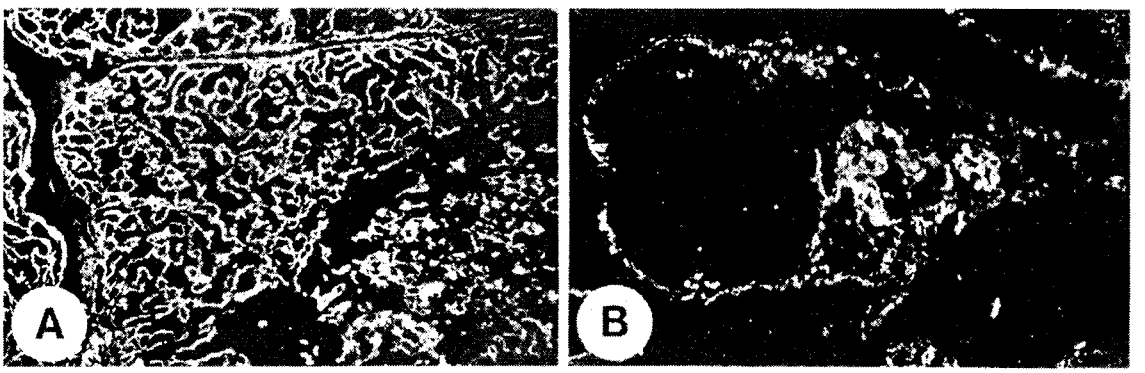

Figure 7. *Immunofluorescence of the thymus epithelial cells.* A: *The control exhibits a dense network within the cortex.* B: *At 66 wpi, most cortical epithelial cells are lost, but some subcapsular and medullary epithelial cells remain. 35βH11, 160×.*

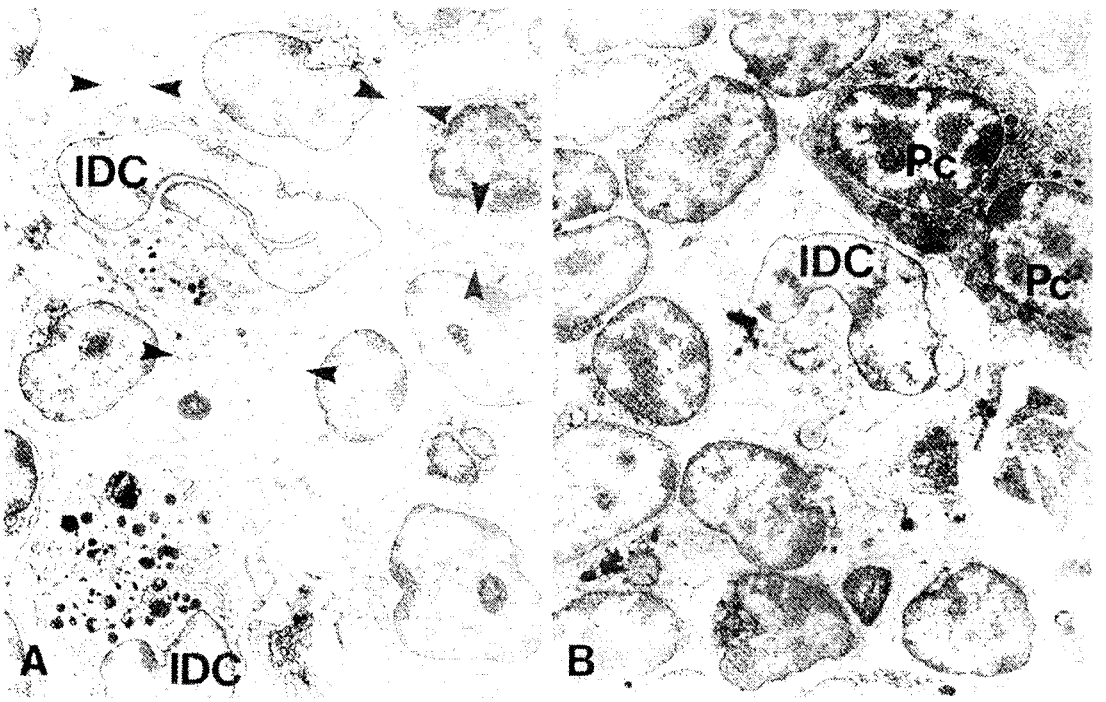

Figure 8. *Interdigitating dendritic cells.* A: *IDC of controls exhibiting long cytoplasmic processes (between the arrowheads) with infoldings of the cell membrane and numerous cytoplasmic organells in the perinuclear cytocentre (3,500×).* B: *IDC at 66 wpi shows complete loss of the cytoplasmic processes and reduced numbers of cellular organelles. Two plasma cells (Pc). 3500×.*

and paraplegia and showed a narrowed thymus cortex comparable with Figure 2C.

Discussion

The thymus was infected by SIV from the first week postinoculation, as demonstrated by positive co-culture experiments, by PCR analysis, and by *in situ* hybridization. The first morphological alteration was a narrowing of the thymus cortex by one-half in the healthy and well-nourished animals at 12 and 24 wpi. The occurrence of the thymus atrophy before the development of immunodeficiency is in accordance with the results of Baskin et al.[16] This early thymus atrophy was not due to stress, malnutrition, or intercurrent infections, but is considered to be SIV-induced.

The unexpected low amount of virus within the thymus of about one copy of proviral DNA in 2×10^4 thymocytes (0.005%) parallels the findings of Baskin.[16] These results were confirmed by immunohistochemistry and by *in situ* hybridization,

Table 4. *Lymph Node Alterations of the Chinese Monkeys in the Time Course of SIVmac251–32H Infection*

Weeks postinfection	controls	1	3	6	12	24	66	122	124
Animal number	14 +16	1	2	3	4	5	6	7	8
Follicular hyperplasia	−/+	+	+++	+++	+++	+++	++	++	−
Altered germinal centers	−	−	−	−	−	+	+	++	+++
Atrophy of paracortex	−	−	−	−	−	−	−	−	+++

−, none; +, slight; ++, moderate; +++, extensive.

which exhibited a positive reaction in a few cells only (1 to 5 signals per slide, each containing about 10^4 to 10^6 cells) and by co-culture experiments. Cell-free supernatants of thymus homogenates failed to produce cytopathogenic effects, in contrast to cell-free supernatants of lymph node homogenates. This indicates the presence of free viral particles in the lymph nodes, but not within the thymus.

The low amount of SIV in the thymus is in contrast to the extrapolations made from *in vitro* infection of human thymocytes.[4–6] It also contrasts results obtained in severe combined immunodeficient mice with an intrathymic HIV-1 infection after transplantation of human thymus and liver.[7] Another retroviral model, the *ts*1 infection of mice, which is considered to resemble HIV infection of the human thymus, resulted in a large scale virus replication in the thymus and in thymus atrophy within a few weeks postinfection.[25] In contrast to these models, the atrophy of the SIV-infected monkey thymus does not seem to be due to a large-scale virus replication, especially because the number of about 0.005% of all thymocytes does not add substantially to the fact that about 80 to 90% of the daily production of thymocytes are programmed to die naturally.[17] One reason for the difference in the results *in vitro* and *in vivo* may be the lack of the immune response *in vitro* allowing an undisturbed virus replication.

In vitro experiments showed direct virus-induced cytopathogenic effects: a remarkable increase of pyknotic cells and the induction of syncytial giant cells. The morphometric evaluation, however, showed that SIV-induced thymus atrophy *in vivo* is not due to an increase in pyknotic cells but on the contrary is characterized by a significant decrease in the rate of pyknotic cells. Syncytial giant cells are a hallmark of the HIV and SIV encephalopathy, but in the thymus, they are a normal finding in non-HIV-infected human beings,[26,27] where they occur in more than 50% of all thymuses.[28] These human thymic giant cells resemble the giant cells in the animals nos. 3 and 4 by morphology and by their localization at the cortico-medullary boundary. In addition, we could not demonstrate any viral particles in the giant cells of the animals nos. 3 and 4, and therefore it remains open whether they were related to the SIV infection or not.

All these results indicate that the SIV-induced thymus atrophy is not due to an increase in thymocyte cell death but is due to an impaired proliferation and maturation of the thymocytes, as shown by the parallel reduction of the mitotic and the pyknotic rate ($r = 0.88$, $P < 0.001$; Figure 4) and by the flow cytometry data showing a relative decrease in the immature double-positive thymocytes of the narrowed cortex. It was a remarkable finding that the relative proportion of CD4+ single-positive thymocytes was increased in all animals with a narrowed cortex, and this gives further support to the hypothesis that SIV-induced thymus atrophy is not due to direct cytopathogenic effects of the virus.

The prerequisite for the intrathymic T cell proliferation and maturation are the cells of the thymic microenvironment: epithelial cells, macrophages, B cells, and interdigitating dendritic cells.[17,26,27,29,30] The ultrastructural alterations of the thymus microenvironment we found in all thymuses with narrowed cortices could have various functional consequences. The cytoplasmic vacuolization in the subcapsular and cortical epithelial cells may indicate some form of a cellular hydrops, but may also reflect an altered secretion of thymus hormones as described in AIDS patients.[31] The loss of the cytoplasmic processes of the cortical epithelial cells (so-called thymic nurse cells[32]) could directly involve the process of positive selection, which depends on a direct physical contact between the thymocytes and major histocompatibility complex class II antigens.[33] These alterations cumulated in cytolysis with a total loss of the cortical epithelium as was shown by cytokeratin immunofluorescence.

This ultrastructural damage of the cortical epithelial cells and the interdigitating cells of the thymus was detected only after a systematic search for the virus, and then a systematic study of the local cells of the thymus was started. This may account for the difference to the study of Baskin who didn't describe alterations of the thymic epithelial cells.[16] A loss of the cortical epithelium was already suggested in thymuses of human AIDS patients,[34,35]

and this indicates that SIV- and HIV-induced thymus atrophy are quite comparable. All these ultrastructural alterations of the cells of the thymus microenvironment could completely explain the breakdown of the intrathymic T cell proliferation and maturation and could therefore be the cause of the SIV-induced thymus atrophy.

SIV-induced thymus atrophy is not a rapid process of a few weeks but seems to start a few months after SIV infection and then progress very slowly. Complete thymus atrophy was seen only in animals with severe AIDS-defining diseases. This time course and its morphological characteristics are quite different from other forms of thymus atrophy. The acute accidental thymus involution induced by cortisone is characterized by an apoptosis of the thymocytes but unaltered thymus epithelial cells.[36] Age-related thymus atrophy shows no alterations of the cortical epithelial cells but is characterized by an increase in the subcapsular fatty tissue.[37,38] In contrast to the first reports of the thymus in HIV infection[39] and in accordance with later reports,[11,40] there was no complete loss of the Hassalls corpuscles but only a reduction in their number.

No virus was detected in the thymus epithelial cells or in the interdigitating dendritic cells (IDC) up to now. *In vitro* infection of human thymus epithelial cells with HIV-1 was reported to be possible by Numazaki et al.[41] but could not be confirmed by Schnittman et al.[42] Other mechanisms causing the necrosis of the cells of the thymus microenvironment might include humoral autoimmune reactions as proposed by Savino et al.[34] They could be triggered by shared epitopes between HIV and various noninfected cells and tissues.[43–45] In immunohistochemistry, this causes frequent cross-reactivities with non-HIV-infected tissues,[11,46–48] and the same may be true with SIV and monkey tissues, as demonstrated in our material. An interesting fact seems to be the cross-reactivity of HIV-1 gp41 with HLA-DR,[49] because all the cell types showing ultrastructural alterations in the SIV-infected monkey thymus are major histocompatibility complex class II-positive. Morphological indications for such a humoral autoimmune mechanism might be the increase in the density of plasma cells in the narrowed monkey thymus and their often close spatial relationship with the altered epithelial cells or IDC. Another possible mechanism that could lead to autoimmune reactions in AIDS patients[50] might be a derangement in the process of negative selection. Thymic interdigitating dendritic cells are involved in the process of negative selection, and their ultrastructural alterations might result in a functional impairment, allowing an escape of unselected autoreactive T-cell clones from the thymus, as was shown in mice treated with cyclosporin A.[51]

What are the possible consequences of the SIV-induced thymus atrophy for the development of the immunodeficiency? The main role of the thymus is to provide the prerequisites for proliferation and education of the prethymic stem cells that come from the bone marrow and go into the periphery.[17] Our data indicate that SIV-induced thymus atrophy interrupted this thymic T cell production and that this SIV-induced thymus atrophy preceded the atrophy of the lymph node paracortex (Tables 3 and 4) as well as the loss of the peripheral T cells.[21] This indicates that the T cell loss in SIV infection starts only after functional knock-out of the thymus. This would furthermore implicate that the treatment of the HIV or SIV infection (if available at all) should start before the irreversible destruction of the thymus microenvironment.

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