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Self Prion Protein Peptides are Immunogenic in Lewis Rats

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Prion diseases are caused by abnormal folding of the prion protein. The paradigm is that the prion protein is not immunogenic because the immune system must be tolerant to such a self protein. In an attempt to identify immunogenic prion peptides, we immunized Lewis rats with peptides that fitted the MHC class II RT1.B^{l} motif. Both humoral and cellular immunity to the prion peptides were obtained without any harmful effects to young animals. However, when 8-month-old rats were immunized, a sixth (6/36) of the rats developed severe skin inflammation with concomitant hair loss. These findings suggest that immunity to self-prion peptides can be readily induced in Lewis rats and that this immune response may have pathogenic consequences in older rats.

Introduction

Prion diseases are a group of central nervous system (CNS) disorders thought to be mediated by the accumulation in the brain of prion protein expressing an abnormal conformation PrP-scrapie (PrP^{Sc}) [1, 2]. Prion diseases, collectively called transmissible spongiform encephalopathies (TSE), have been characterized in various animal species [3]. In humans the disease can result from a genetic mutation [4] or from 'infection' with the abnormally folded protein [5]. Following ingestion, PrP^{Sc} is absorbed from the gastrointestinal tract and transferred to the CNS [6].

The leading hypothesis regarding the pathogenesis of prion diseases is that the abnormal conformation serves as a template for the continued formation of itself. Accumulation of the abnormal protein leads to the death of neurons [6].

The immune system is not considered to participate in the molecular evolution of prion disease [7]. However, recent studies in B cell knockout mice have demonstrated that the progression from peripheral inoculation of PrP^{Sc} to prion disease is dependent on B cells [8, 9], and it was concluded that B cells can serve as vehicles for the transfer of PrP^{Sc} from the periphery to the CNS. Attempts to induce antibodies to self-PrP have not been successful [7]. Indeed, monoclonal anti-PrP antibodies were produced in PrP knockout mice because normal mice, which express prion protein, were considered to be tolerant to this molecule [7].

Our group has characterized the MHC class II motifs of the Lewis rat [10]. Peptides that fit this motif were found to be immunogenic [11]. The aim of the present study was to use the MHC II motif to select candidate peptides from rat prion protein and to use these peptides to investigate the potential induction of an autoimmune response. We found that Lewis rats developed both T cells and antibodies specific to prion peptides. Immunization of young rats (2–6 months of age) did not induce any demonstrable pathology; however, rats immunized at 8 months of age developed skin lesions. These findings modify our concepts regarding the ability of the immune system to react to prion protein peptides. The effect of prion autoimmunity on the pathogenesis and treatment of scrapie is an important subject for investigation.

Materials and Methods

Animals

Inbred female Lewis rats were supplied by Harlan Olac, Bicester, UK or were obtained from the Weizmann Institute breeding colony. The rats were maintained in an SPF facility.

Peptides

The rat prion protein sequence was searched by visual inspection for peptides that fit the motif we reported

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for the Lewis rat (RT1.B^{l}) MHC class II molecule [10]. Three suitable prion peptides termed p118–137, p182–202, and p211–230 were detected. Peptides were synthesized by using the protocol for N-a-flourenylmethoxycarbonyl (Fmoc) synthesis, using an automated ABIMED synthesizer AMS422 (Langenfeld, Germany) as described [12]. The peptides were tested for purity using HPLC and their composition was confirmed by amino acid analysis.

Immunization

Naive female Lewis rats were immunized in each hind footpad with 50 µl of emulsion containing 25 µg of peptide; each rat received a total of 50 µg of peptide. Complete Freund's adjuvant (CFA) was prepared by mixing incomplete Freund's adjuvant with 4 mg/ml of *Mycobacterium tuberculosis* H37Ra, and the peptides were dissolved in PBS (1 mg/ml) and emulsified with CFA at a 1:1 (v/v) ratio.

T cell lines

Draining popliteal lymph node (LN) cells were removed on day 10 after immunization, and single cell suspensions were prepared by pressing the organs through a fine wire mesh. To establish antigen-specific T cell lines, LN cells were stimulated with the immunizing peptide (5 µg/ml) for 3 days in stimulation medium [DMEM supplemented with β mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 u/ml), streptomycin (100 µg/ml), non-essential amino acids (1 ml/100 ml), and autologous serum, 1% (v/v)] [13]. Following stimulation, the T cell blasts were seeded in propagation medium (identical to stimulation medium without autologous serum, but supplemented with 10% fetal calf serum), and 10% (v/v) supernatant of Con A-stimulated spleen cells, containing T cell growth factors [13]. Five days after seeding, the cells (5×10^{5}/ml) were re-stimulated with the peptide (5 µg/ml) and irradiated thymocytes as antigen presenting cells (10^{7}/ml) for 3 days in stimulation medium. Lines were expanded by repeated stimulation with peptides and irradiated thymocytes every 8–10 days [14], for the number of cycles indicated. The cells were analyzed for their specificity to immunizing peptides in a proliferation assay at each stimulation cycle. Each line was derived from the pooled lymph node cells of two rats in each group. Each experiment using p118–137 and p182–202 was repeated three times.

T cell proliferation assay

T cell proliferation assays were performed by culturing 5×10^{4} cells (from each line) with irradiated (2500R) thymocytes as antigen presenting cells (2.5×10^{6}/ml) in stimulation medium. Cells were incubated for 3 days at 37°C in humidified air containing 7% CO_2 in the presence of 1 µg/ml, 5 µg/ml, or 50 µg/ml peptide, or 10 µg/ml ovalbumin or 1.25 µg/ml Con A as negative and positive controls, respectively. The assay was performed in a 96 microtiter (Nunclon™-Delta-surface, Denmark). Each well was pulsed with 1 µCi of (^{3}H) thymidine (5.0 Ci/mmol, Amersham, Buckinghamshire, UK) for the final 18 h. The cultures were then harvested using a Micro-Mate 96 Cell Harvester and cpm were determined using a Matrix 96 Direct Beta Counter using avalanche gas (98.7% helium; 1.3% C_4H_{10}) ionization detectors (Packard Instrument Company, Meriden, CT, USA).

Determination of anti-prion peptide antibody

Immunized rats were bled 10 weeks after immunization, and their sera were collected. Control rats were injected with PBS/CFA. The antibody response to the relevant PrP peptide was measured using a standard ELISA assay [15].

Flow cytometry of the T cell line

Line cells were collected after their sixth stimulation and on their third day of rest. The cells were washed with 1% FCS and 0.02% Na-azid in PBS (FACS buffer). Subsequently, the cells were centrifuged (1000 rpm) and resuspended in the FITC conjugated antibody (Pharmingen, San-Diego CA, USA), and incubated for 45 min at 4°C in the dark, and samples were analyzed using the FACScan (Becton Dickinson, New Jersey, USA). The cells were tested for surface expression of CD4, CD8, αβTCR, Vβ 8.2, Vβ 8.5, Vβ 10, and Vβ 16. Analysis of the results was done using the Cell Quest software package.

Histology

Rat skin was removed and fixed in 10% formalin. Then the tissue was embedded in paraffin and 5 µm sections were cut by microtome, and stained with hematoxylin and eosin (H&E).

Results

B cell responses to the prion peptides

Applying our previous findings of the MHC class II binding motif of the Lewis rat (RT1.B^{l}) [10], we synthesized 20–21 mer peptides of the prion protein to identify potentially immunogenic peptides. We detected three peptides that contained a suitable motif. These peptides were termed p118–137, p182–202, and p211–230 (Table 1).

Groups of 8-month-old female Lewis rats were immunized with p118–137 or p182–202 (Table 1). Age-matched unimmunized female Lewis rats were used as controls. To examine whether the prion peptides induced antibodies, Lewis rats were bled ten weeks after being injected with p182–202 or p118–137 peptides in CFA. Figure 1 shows that both peptides

Table 1. *The sequence of the prion peptides from the rat prion protein*

Prion peptide		Amino acid sequence MHC anchor positions 1	2	**3**	**4**	5	**6**	7	8	**9**	
p118–137	AGAVVGGLG	G	Y	**M**	**L**	G	**S**	A	M	S	RP
p182–202	ITIKQH	T	V	**T**	**T**	T	**T**	K	G	**E**	NFTETD
p211–230	Q	M	C	**V**	**T**	Q	**Y**	Q	K	**E**	SQAYYDGRRS
p101–120		P S K P K T N L K H V A G A A A A G A V									
p31–50		W N T G G S R Y P G Q G S P G G N R Y P									

Bold letters indicate anchor positions 3, 4, 6 and 9 of prion peptides in the Lewis rat RT1B^{l} MHC class II [10]. Control peptides p101–120 and p31–50 have no anchor positions for the RT1Bl MHC class II molecule.

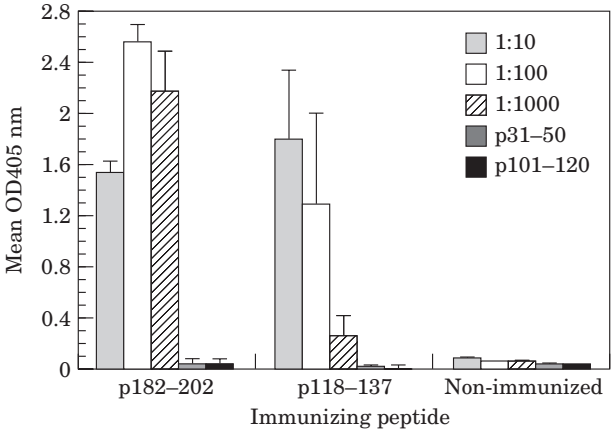

Figure 1. Antibodies to prion peptides. Groups of Lewis rats were immunized with peptides p182–202 or p118–137. Control rats were not immunized. Sera were collected 10 weeks later and tested for IgG antibodies to the specific peptide at three dilutions, and for antibodies to control prion peptides p31–50 or p101–120 at a dilution of 1:100. The bars represent the mean+SD of the OD at 405 nm.

p182–202 and p118–137 induced a specific antibody response to the immunizing peptide and not to irrelevant prion peptides p31–50 or p101–120 (Table 1); p182–202 was more immunogenic compared to p118–137.

T cell responses to the prion peptides

Primary proliferation was induced by p118–137, p182–202, and p211–230, but not by p101–120 (data not shown). To analyze the T cells, LN cells were pooled and T cell lines were generated by repeated *in vitro* stimulation with the peptide and antigen presenting cells. Lines p182–202 and p211–230 showed strong specific proliferation (Figure 2). The proliferation of the line p118–137, however, was less strong. Line p211–230 appeared to decline after the third stimulation cycle (data not shown), and we decided to focus on line p182–202 for passive transfer and marker studies. Cycles of stimulation and rest were continued to the sixth stimulation and the specificity of the p182–202 line was measured against a panel of myelin basic protein (MBP) peptides [16]. We found that the

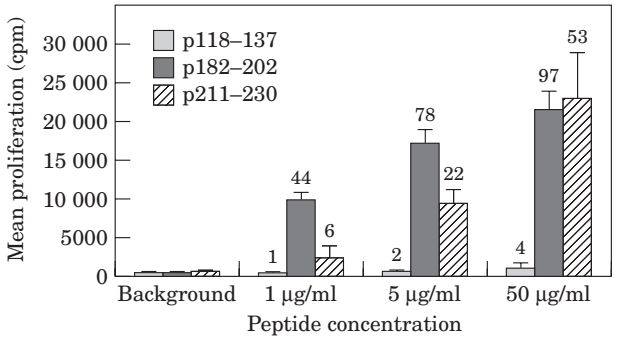

Figure 2. Proliferative responses of p118–137, p182–202 and p211–230 T cell lines. The proliferation is shown after the second stimulation as the mean CPM and the stimulation index (SI; cpm of experimental wells divided by cpm of wells without antigen). The numbers on top of each column indicate the stimulation index. The bars represent the mean+SD of the mean proliferation.

p182–202 line was highly specific for the immunizing p182–202 peptide and not for irrelevant autologous MBP peptides or for ovalbumin (data not shown).

FACS analysis of the p182–202 T cell line

The p182–202 T cell line was further characterized by FACS, and the percentage of cells expressing CD4, CD8, αβTCR, Vβ 8.2, Vβ 8.5, Vβ 10 and Vβ 16 are shown in Figure 3. We found that the majority of the cells were CD4 positive and expressed αβTCR. Among the four Vβs examined, the line showed a higher proportion of cells expressing Vβ16.

Active immunization to PrP peptides can be pathogenic

To learn whether active immunization to PrP peptides might be pathogenic, we continued to observe groups of immunized rats for over a year. We found that the young rats (age 2–6 months) did not develop any disease, even when they had been immunized to the prion peptides together with an injection of 200 ng of pertussis toxin (data not shown). However, two of the eight rats immunized at the age of 8 months and two

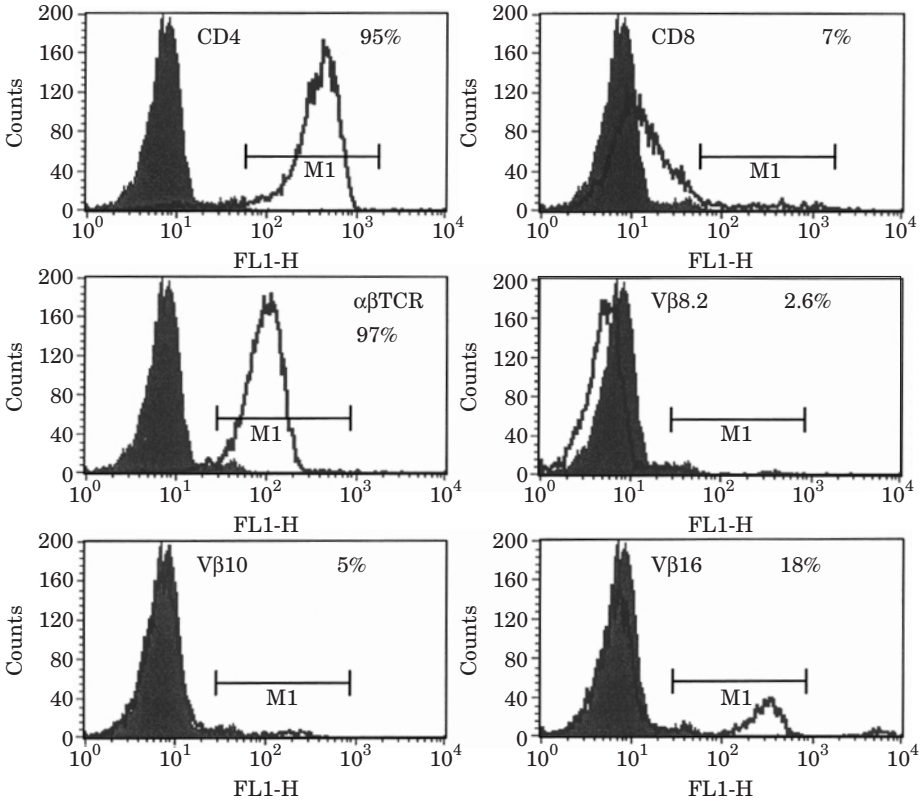

Figure 3. FACS analysis of the p182–202 T cell line. The line was studied at the sixth stimulation, after three days of rest. Anti-p182–202 line cells were incubated with various monoclonal antibodies to CD4, CD8, αβ TCR, Vβ 8.2, Vβ 10, and Vβ 16, and tested by FACScan analysis. The percentage of cells stained with each antibody is indicated in the histograms.

out of thirteen rats immunized at 6.5 months with p182–202 developed severe skin inflammation, and loss of hair 8 to 12 months after immunization. Figure 5 compares the skin of a sick rat to that of an age-matched control rat. It can be seen at low magnification (upper panels) that the sick rat manifested extensive mononuclear infiltrates around hair follicles and throughout an edematous, thickened dermal area. The higher magnification (lower panels) shows invasion and destruction of the hair follicles. Injected rats that did not develop clinical dermatitis showed no inflammatory infiltration in the histological examination (not shown). Figure 4 compares a sick rat with a healthy, age-matched control rat. Immunized rats that did not develop dermatitis appeared identical to the healthy control rat. Skin inflammation was seen in two out of fifteen Lewis rats immunized at 6.5 months with p211–230 (data not shown).

In addition, the splenocyte reactivity was tested in a proliferation assay to the p182–202 as well as to a panel of MBP peptides [16]. We found that splenocytes from the sick rat were still responsive for the immunizing p182–202 peptide (Figure 6A) and not to an irrelevant autologous MBP peptides (data not shown). Nevertheless, splenocytes from the control rat did not respond to either the p182–202 or the MBP peptides, but did respond well to non-specific control stimulation with Con A (data not shown).

To investigate the possible role of anti-p182–202 humoral response in the observed pathology, sera from the bald sick rat as well as sera from a control rat immunized with CFA were tested in an ELISA assay against p182–202 or to the irrelevant prion peptide p101–120 (Table 1). We found that the sick rat sera had persistent IgM and IgG antibodies specific to p182–202 but not to the irrelevant p101–120 peptide after ten months of immunization. The anti-p182–202 antibodies were present in the sera of the sick rat and not in the sera of the CFA-control rat (Figure 6B). Sera from rats immunized with p182–202 but did not show skin pathology were also positive for antibody reactivity 8–10 months after immunization (data not shown).

Discussion

Much work on the prion protein has focused on the molecular pathogenesis of TSE [17–24]. Studies of the role of the immune system in prion disease have examined the contribution of various cellular components to the peripheral replication of the abnormal prion protein and its transportation to the CNS [25]. These studies concluded that T cells do not participate in the transport of prions to the CNS, while both B cells and follicular dendritic cells are critical for this process [25–27]. To our knowledge, a specific immune response to self-prion protein or its peptides has not been previously reported.

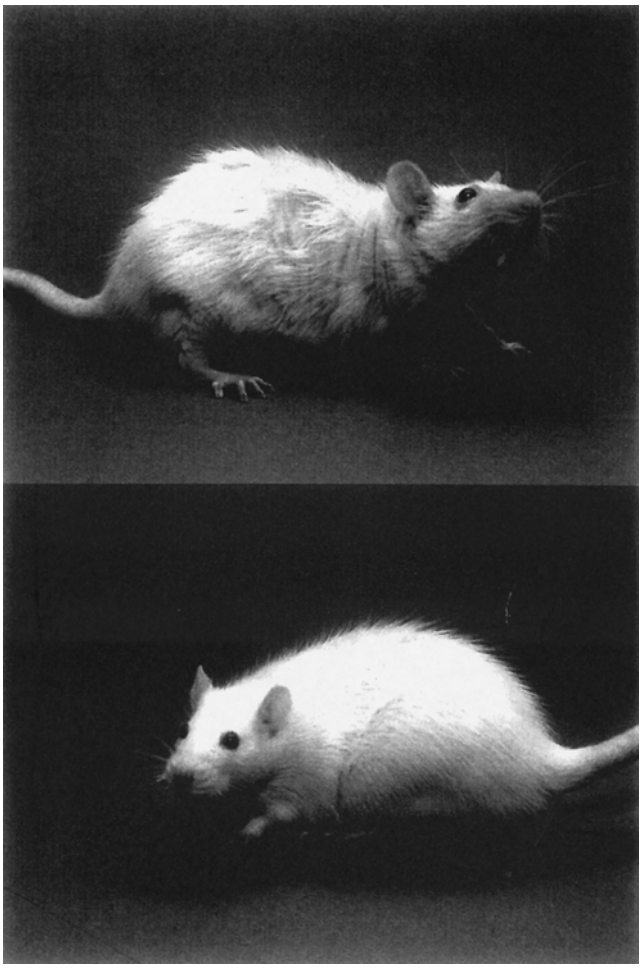

Figure 4. A sick rat (upper frame) and a healthy rat (lower frame), both around 18 months old. The sick rat was immunized to p182–202 at the age of 8 months and the healthy rat was not immunized.

We here report that immunizing Lewis rats with synthetic self-prion peptides that contain the specific MHC class II ($RT1.B^{l}$) motif induced both a T cell response and antibodies (Figures 1 and 2). The ability to raise an immune response to several prion peptides indicates that, contrary to the prevailing paradigm [7, 28], the immune repertoire is not depleted of T or B cells reactive to prion epitopes. This raises the possibility that such immune cells might arise during the natural history of prion disease and affect the disease process. This question should, in the light of our findings, be studied. Unfortunately, because of biohazard regulations in our institute, we could not test whether the T cells or antibodies we raised to the peptides could recognize the intact prion protein, either in its native or aberrant conformations.

Pathological changes in the skin were seen in some of the rats immunized with p182–202 and p211–230 in 6.5 and 8-month-old rats but not in younger rats. We found that the older the rat is the more susceptible it becomes to the prion-induced skin pathology. The percentage of susceptibility to skin lesions increased from ~15% in 6.5-month-old immunized rats to 25% in 8-month-old immunized rats with p182–202. In addition, the time interval for the lesions to occur was longer in younger rats. However, because of the length of time between immunization and skin disease, it is conceivable that the changes could have resulted from an unrelated pathogenic mechanism. However, pathologic skin inflammation has not been seen in control animals of the same experiment nor in our animal facility in any of the hundreds of Lewis rats kept in the same conditions. Furthermore, the skin histology was negative for skin parasites using PAS staining (data not shown). The rats that developed lesions were housed in separate cages with rats that did not develop dermatitis; therefore, the skin disease was apparently not contagious. Thus, it is possible that prion autoimmunity may be pathogenic in certain circumstances. However, the pathogenic mechanism responsible for these changes is not clear. In most experimental models of autoimmune diseases, the pathology usually appears within 10–14 days post immunization [16]. Rarely, the clinical symptoms may be delayed for several months [29]. A long period of incubation could result from the need to induce a pathogenic population of effector cells, or the need to overcome strict regulatory mechanisms. The mechanism through which the immune response to p182–202 participates in the observed pathology is unclear. Histological examination of the skin clearly demonstrated inflammation and invasion of lymphocytes in the hair follicles (Figure 5), which progressed to involve large areas of the skin within a few weeks (Figure 4). Attempts to transfer the skin disease to naïve rats by inoculation of a p182–202 specific T cell line or by Con A-activated spleen cells from a sick rat to either naïve or irradiated rats were not successful (data not shown). In addition, we examined the antibody responses and primary splenocyte T cell proliferation in p182–202 immunized rats that developed pathological signs, and compared the immune responses to those of rats that were immunized with the same peptide and did not develop any pathology. We found that rats from both groups demonstrated antibody responses to the p182–202 peptide, however, T cell proliferation was seen only in the sick rats. Thus, it is possible that T cell immunity to p182–202 plays some role in disease induction. It is noteworthy that the skin expresses the prion protein [30, 31]. This suggests that the observed pathology might be due to autoimmunity to p182–202. Unfortunately, the long incubation period between immunization and disease is a serious obstacle to detailed investigation. But we report our observations so that interested parties may take note.

Recent work on a mouse model of Alzheimer's disease has shown that immunization with a beta-amyloid peptide can prevent the accumulation of amyloid in the brain [32, 33]. Amyloid formation is also part of the pathogenic mechanism in prion diseases [34], and our work suggests that antibodies to prion peptides should be investigated for their possible influence on amyloid formation in the prion diseases.

In summary, our work shows that self PrP peptides can elicit both cellular and humoral immune

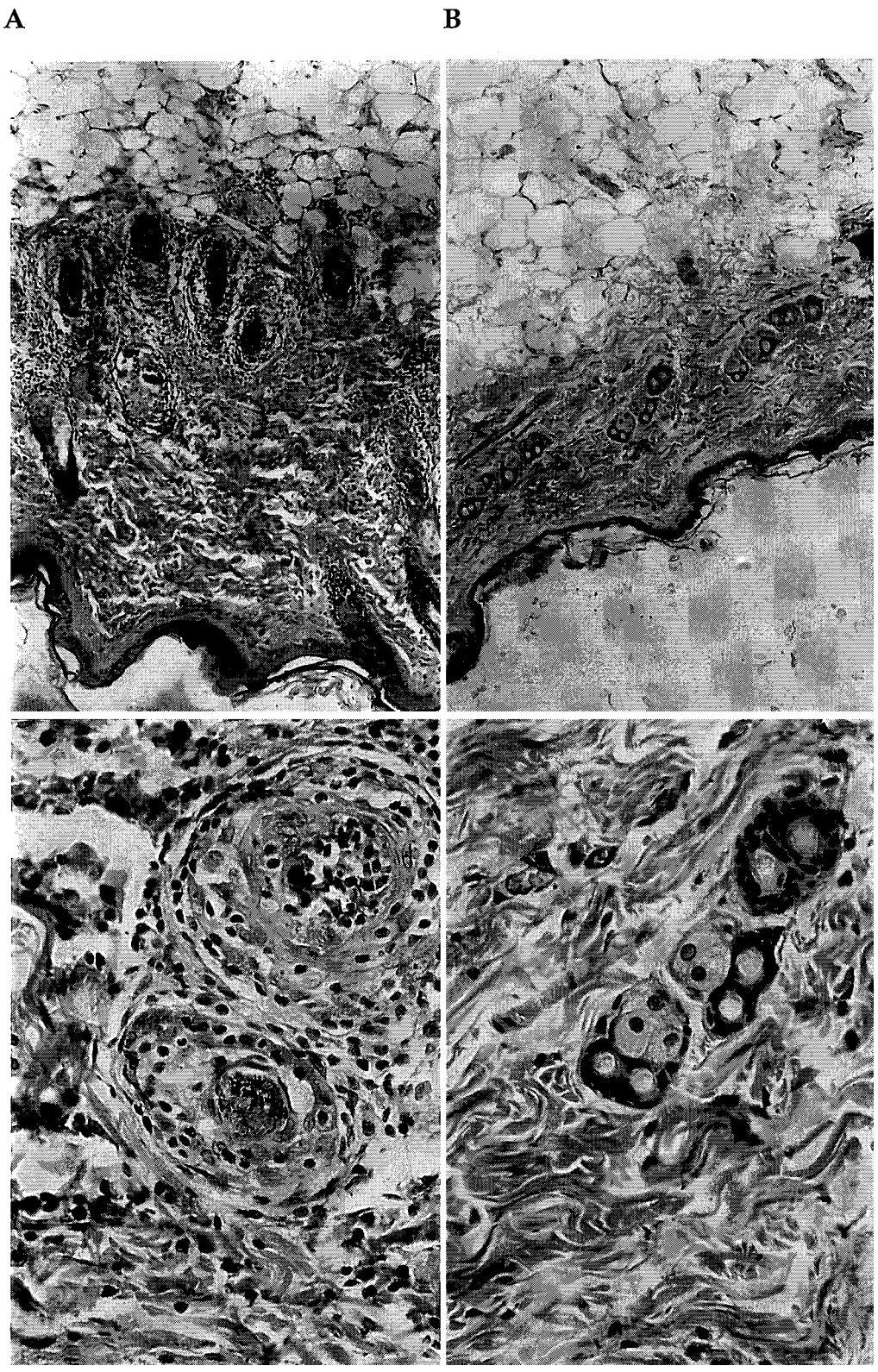

Figure 5. Skin histology of sick and control rats. The rats described in the legend of Figure 4 were sacrificed and their skin was studied histologically. The sections were stained with H&E, and pictures were taken at magnifications ×100 (upper frames) and ×400 (lower frames). (A) Skin sections from sick rat. (B) Skin sections from healthy rat.

responses in Lewis rats. We also provide evidence that prion peptide immunoreactivity can cause skin lesions due to mononuclear cell infiltration to the dermis and epidermis regions. The significance of such immunization on the natural history of prion disease as well as its ability to prevent scrapie prion accumulation should be explored in the future.

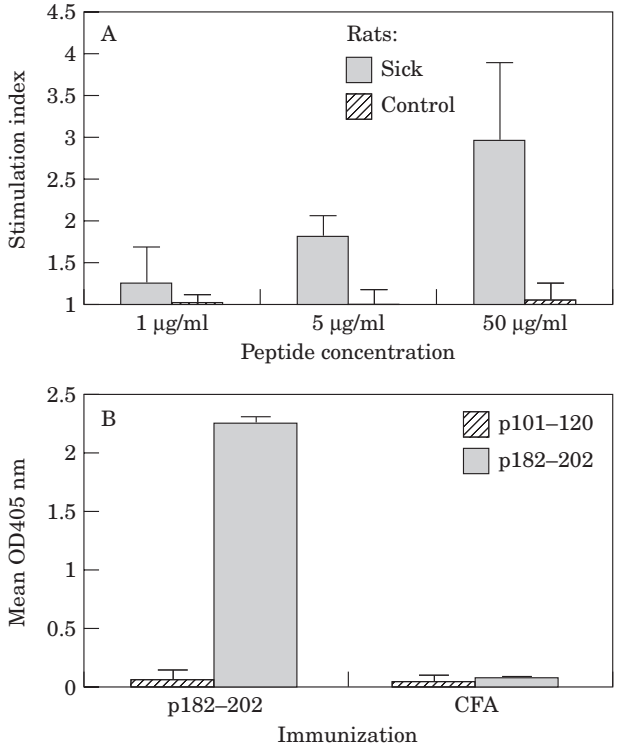

Figure 6. T cell and antibody reactivity in sick rats. Rats, 8 months old, were immunized with p182–202 and observed 10 months later for the persistence of T cell proliferation and antibodies. Control rats were either not immunized or were immunized with CFA alone. (A) Proliferative responses of splenocytes from a sick rat and an age-matched non-immunized rat to the p182–202 peptide. The proliferation is shown as the stimulation index. (B) The amount of IgG antibody (mean+S.D.) in serum diluted 1:10 from the sick Lewis rat compared to an age-matched control rat immunized with CFA, as measured by ELISA.

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