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Spontaneous development of organ-specific autoimmune lesions in aged C57BL/6 mice

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SUMMARY

We have shown that spontaneously occurring, organ-specific autoimmune lesions develop in aged C57BL/6 mice of both sexes, especially in 24-month-old senescent mice. The inflammatory lesions were found in the multiple organs such as salivary gland, kidney, pancreas, lung, and liver, associated with ageing process. Organ-specific autoimmune lesions first appeared in 6-month-old C57BL/6 mice, and were aggravated with advancing age. In contrast, significant inflammatory changes did not develop in the thyroid, stomach, testis, ovary, and prostate in aged C57BL/6 mice. The incidence and severity of organ-specific autoimmune lesions in this strain of non-autoimmune mice increase with advance of age. The most severely affected lesion was sialadenitis developed in the submandibular salivary gland of aged mice, and a significant difference between male and female mice was noted only in the salivary gland. The infiltrating cells within the lesions of multiple organs consisted mainly of Thy 1.2^+ and $L3T4^+$ cells. Autoantibodies were detected in the sera of the mice with each corresponding organ-specific autoimmune lesions.

Keywords immunohistochemistry autoimmune lesions ageing T lymphocytes circulating autoantibodies

INTRODUCTION

The decline in immunologic activities in aged mice appears to be responsible for the increase in the incidence of autoimmune diseases besides malignant neoplasms and infectious diseases (Walford, 1969; Makinodan & Kay, 1980; Hirokawa, 1985). In a previous communication, we reported the spontaneous occurrence in ageing BDF1 mice of an autoimmune sialadenitis that has similarities to Sjögren's syndrome in humans (Hayashi *et al.*, 1988). The infiltrating cells in the lesions of salivary glands in aged BDF1 mice consisted mainly of Thy 1.2^+, $L3T4^+$ cells.

Several strains of autoimmune disease-prone mice have been useful as animal models for the study of human autoimmune disorders (Howie & Simpson, 1976; Kyogoku, 1977; Andrews et al., 1978; Murphy, 1981; Hang, Theophilopolous & Dixon, 1982; Theofilopoulos & Dixon, 1985). Although the spontaneous occurrence of lymphocytic infiltration in several organs similar to organ-specific autoimmune diseases in humans has been reported in these autoimmune disease-prone mice (Kessler, 1968; Greenspan *et al.*, 1974; Takeda & Ishikawa, 1983; Hoffman *et al.*, 1984), spontaneously occurring organ-specific autoimmune lesions in non-autoimmune prone mice have not yet been reported. Thus, we were interested whether organ-specific autoimmune lesions as well as sialadenitis may develop spontaneously in any strain of non-autoimmune disease prone mice associated with ageing process.

We describe here spontaneously occurring organ-specific autoimmune lesions in the multiple organs such as salivary gland, lung, pancreas, liver, and kidney in aged C57BL/6 mice which show an apparent T cell-dependent immunologic disorders with advancing age.

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MATERIALS AND METHODS

Animals

Female and male non-lpr strain of C57BL/6NCrj (B6) mice at 4 weeks of age were purchased from Charles River Japan Inc., (Atsugi, Japan). The mice were reared in our specific pathogen-free (SPF) mouse colony throughout their life. Their mean life-span used for the present study was 21·6 months in females and 22·2 months in males. At 3, 6, 12, 18, and 24 months of age, B6 mice of both sexes (a total of 146 mice) were killed and provided for histological and immunohistological examinations.

Histology

All organs were removed from the killed mice, fixed with 4% phosphate-buffered formaldehyde (pH 7·2) and prepared for histologic examination. The sections were stained with haema-

Table 1. Spontaneous development of organ-specific autoimmune lesions in aged C57BL/6 mice

Age (month)	Sex	*n*	Sial adenitis	Renal lesion	Lesion of islet	Pulmonary lesion	Cholangiolitis
3	Female	10	0	0	0	0	0
	Male	10	0	0	0	0	0
6	Female	10	1(10·0)	0	0	1(10·0)	0
	Male	10	0	1(10·0)	0	0	0
12	Female	13	4(30·8)	4(30·8)	4(30·8)	3(23·1)	3(23·1)
	Male	12	3(25·0)	3(25·0)	4(33·3)	3(25·0)	2(16·7)
18	Female	15	9(60·0)	6(40·0)	7(46·7)	5(33·3)	4(26·7)
	Male	15	7(46·7)	5(33·3)	6(40·0)	6(40·0)	5(33·3)
24	Female	26	23(88·5*)	18(69·2)	15(57·7)	14(53·8)	13(50·0)
	Male	25	17(68·0*)	14(56·0)	15(60·0)	13(52·0)	13(52·0)

Number of mice with organ-specific inflammatory lesions including mild to severe ones. Mild inflammatory lesion indicates that more than 50 infiltrating mononuclear cells are observed microscopically at the middle part of each organ under × 100 magnification. See Materials and Methods for the details.

Numbers in parentheses indicate percent incidence: the proportion of numbers of mice with inflammatory lesions to the total number of mice examined.

* Difference statistically significant at $P < 0.05$ (Mann–Whitney *U*-test).

toxylin and eosin (H&E) and periodic acid-Schiff (PAS) method. Mild inflammatory lesion in each organ indicates one or two foci per representative longitudinal section. In this case 'focus' means mononuclear cell infiltration, including lymphocytes, plasma cells, and macrophages composed of more than 50 cells at the middle part of the organs, under × 100 magnification. Moderate lesion indicates three to five these inflammatory foci, and severe lesion indicates over five foci of mononuclear cell infiltration showing in separate and/or in contiguous manner with a considerable degree of parenchymal destruction.

Immunohistologic staining

Immunohistologic staining with monoclonal antibodies were performed on freshly frozen sections utilizing biotin–avidin immunoperoxidase method. Briefly, frozen sections approximately 4 μm in thickness were fixed in acetone and rinsed in cold phosphate-buffered saline (PBS, pH 7·2), and incubated with the commercially available blocking kit (Vector Laboratories, Burlingame, CA) for 20 min. They were then incubated for 1 h with each of biotinylated rat monoclonal antibodies to Thy 1.2, L3T4 and Lyt2 (Becton-Dickinson, Sunnyvale, CA; L3T4 was biotinylated in our laboratory) at a dilution of 1:80, respectively. They were washed with cold PBS for 30 min and incubated with avidin and biotinylated horseradish–peroxidase complex (ABC reagent, Vector Laboratories) for 30 min. After washing three times with PBS, the sections were reacted with a fresh mixture of 0·05% 3, 3′,-diaminobenzidine and 0·005% H_2O_2 in Tris-HCl buffer (0·05 M, pH 7·6) for 5 min, washed with distilled water, and were lightly counterstained with haematoxylin. Negative controls were carried out with normal rat serum (Cappel Laboratories, Cochranville, PA) or PBS instead of initial incubation. To detect the B cell lineage, the sections were incubated with rabbit serum to mouse immunoglobulins (Dakopatts, Copenhagen, Denmark) at a dilution of 1:60 for 30 min at room temperature. Thereafter, anti-rabbit IgG labelled with horseradish peroxidase (Miles Laboratories, Elkhart, IN) was applied for 30 min at room temperature. The peroxidase was localized in the same manner as described above.

Detection of autoantibody

The presence of autoantibodies in sera from individual mice was examined by the indirect immunofluorescent (IF) antibody staining technique, as described previously (Hayashi, Sato & Hirokawa, 1985; Hayashi *et al.*, 1986; 1988). Briefly, cryostat sections of salivary gland, thyroid, thymus, lung, stomach, liver, pancreas, kidney, brain, heart, adrenal, ovary, testis, prostate and seminal vesicle of normal young (2-month-old) B6 mice were first washed with cold PBS to remove endogenous immunoglobulings, fixed with acetone for 5 min and rinsed with cold PBS, and then used as target antigens for indirect IF assays. Samples were randomly chosen from each age group of mice with or without inflammatory lesion. Sera diluted 20-fold were used for testing autoantibodies. The serum titre of autoantibody was determined at the maximum dilution giving positive reaction on frozen section.

RESULTS

Histological study

Table 1 summarizes the incidence of the inflammatory lesions (mild to severe) developed in multiple organs such as salivary gland, pancreas, kidney, lung, and liver, in B6 mice at various age group. The first detectable changes in H&E sections were observed at 6 months of age, when mild periductal mononuclear cell infiltrates in the submandibular salivary gland, mild perivascular infiltrates in the lung, and mild mononuclear cell infiltrates in the interstitium of the kidney were noted. At 12 months of age, mild to moderate mononuclear cell infiltrates were observed in the pancreatic islets and periductulal area of the portal triads as well as in the salivary gland, kidney and lung. The incidence and severity of spontaneously occurring inflam-

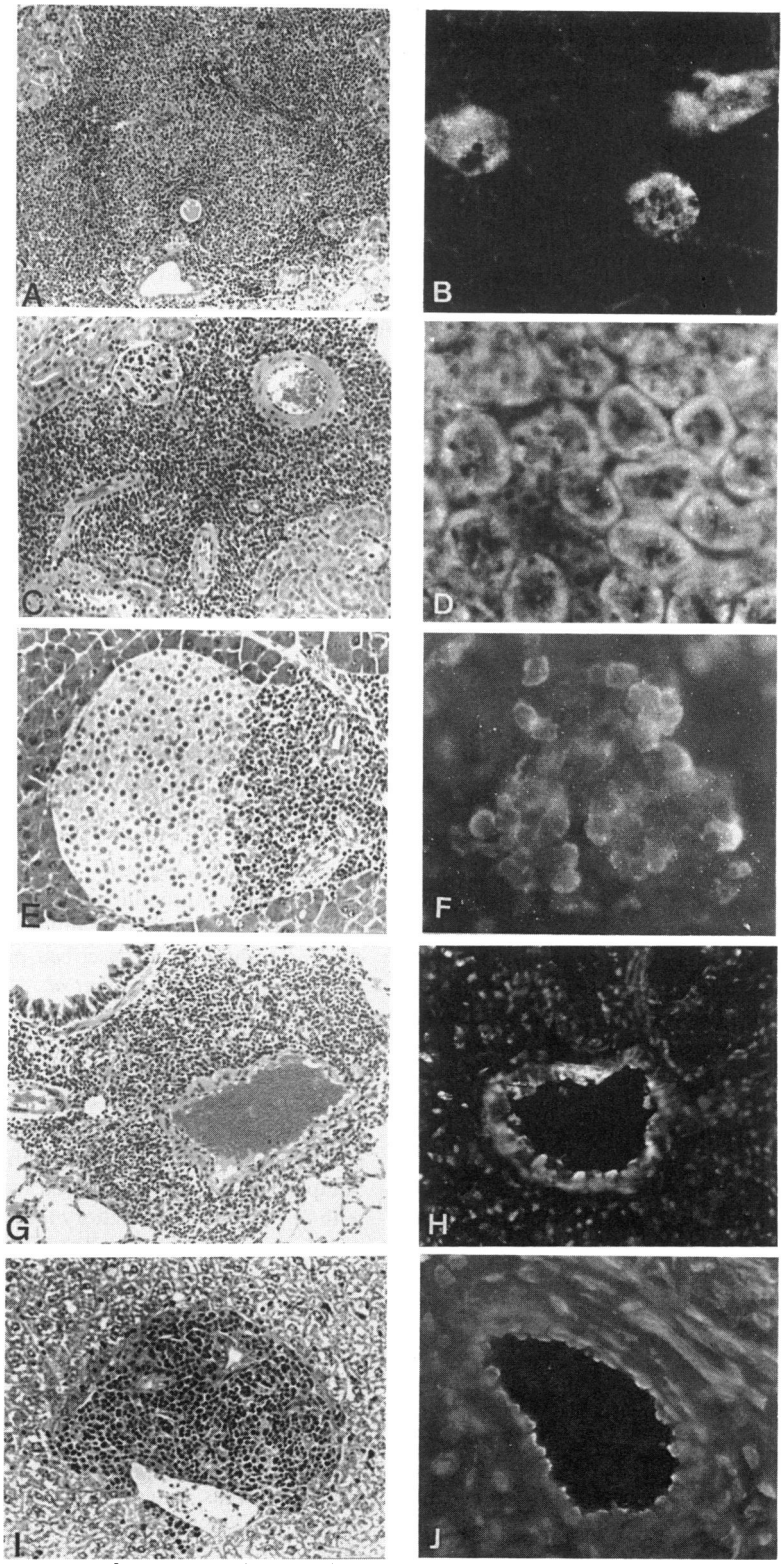

Fig. 1. Histologic appearances of spontaneously occurring organ-specific autoimmune diseases and demonstration of their corresponding autoantibodies in 24-month-old C57BL/6. (A) Severe destructive lesion of submandibular salivary gland with extensive infiltration of mononuclear cells, and (B) detection of anti-salivary duct antibody. (C) Focal interstitial inflammatory cell infiltrate in renal corticomedullary zone, and (D) detection of anti-tubular epithelial cell antibody. (E) Moderate mononuclear cell infiltrate in pancreatic islet, and (F) detection of anti-islet cell antibody. (G) Focal perivascular infiltrate of mononuclear cells in the lung, and (H) detection of anti-bronchiolar epithelial cell antibody. (I) Periductular inflammatory focus in the portal triad of the liver, and (J) detection of anti-bile ductulal cell antibody. (Staining and magnification: haematoxylin and eosin—A, × 48; C, × 160; E,250; G, × 160; I, × 160; IF staining—B,F,H, and J, × 290; D, × 220).

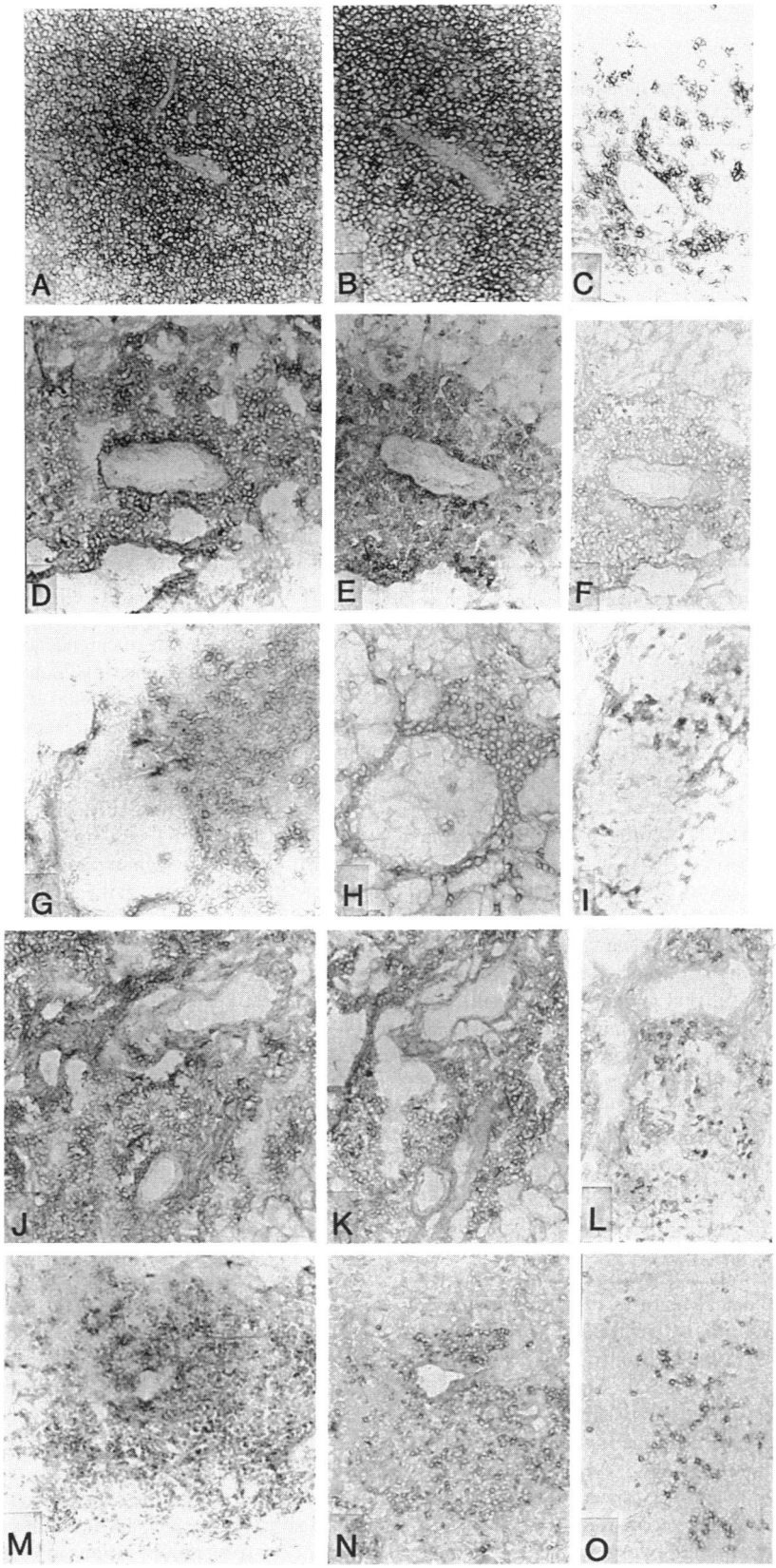

Fig. 2. Immunohistochemical detection of infiltrating lymphocytes in multiple organs such as submandibular salivary gland (A–C), kidney (D–F), pancreas (G–I), lung (J–L), and liver (M–O) in 24-month-old C57BL/6 mice. Sialadenitis of a 24-month-old female C57BL/6 mouse stained serially with Thy 1.2 (A); L3T4 (B); and Lyt2 (C) (× 120). Interstitial renal lesion of a 24-month-old male C57BL/6 mouse stained serially with Thy 1.2 (D); L3T4 (E); and Lyt2 (F) (× 120). Lesion of pancreatic islet of a 24-month-old male C57BL/6 mouse stained serially with Thy1.2 (G): L3T4 (H); and Lyt2 (I) (× 240). Pulmonary vasculitic lesion of a 24-month-old female C57BL/6 mouse stained serially with Thy1.2 (J); L3T4 (K); and Lyt2 (L) (× 120). Cholangiolitic lesion of a 24-month-old male C57BL/6 mouse stained serially Thy1.2 (M); L3T4 (N); and Lyt2 (O) (× 120).

Table 2. Immunohistochemical analysis of inflammatory lesions in 24-month-old C57BL/6 mice

Organs	Mean percent* ±s.d. (range in parentheses) of monouclear cells staining with			
	Thy1.2	L3T4	Lyt2	sIg
Salivary gland	86±6 (78–94)	81±7 (74–91)	10±3 (4–14)	7±2 (4–11)
Kidney	84±7 (74–92)	75±7 (64±84)	9±3 (6–14)	5±1 (3–7)
Pancreatic islet	88±6 (77–93)	74±7 (64–82)	8±3 (3–11)	4±2 (2–7)
Lung	81±6 (72–89)	71±10 (54–82)	11±4 (5–16)	9±3 (5–14)
Liver	83±6 (74–91)	75±9 (61–88)	10±4 (4–17)	8±2 (6–12)

* The percentage of mononuclear cells staining positively with a given monoclonal antibody was enumerated using a 10 × 20 grid net micrometer disc, covering an area of 0·16 mm² objective.

Range of seven samples examined.

sIg, surface immunoglobulin.

matory lesions in multiple organs increased gradually with advance of age in both sexes. In particular, the highest incidence of these lesions was observed at 24 months of age in all organs. The most severely affected lesion was sialadenitis, developed in the submandibular salivary gland of aged B6 mice (60% in females aged 18 months; and 88·5% in females, 68% in males aged 24 months). Widespread mononuclear cell infiltrate of the glands with extensive parenchymal destruction was frequently noticed in the submandibular salivary glands in aged B6 mice. A significant difference was noted between male and female mice only in the salivary gland lesions at 24 month of age ($P < 0{\cdot}05$, Mann–Whitney *U*-test). In renal lesions, a variable degree of focal lymphocytic infiltrates was observed in the interstitium, being usually located in the corticomedullary zones in aged B6 mice. There was not histological evidence showing fibrinoid necrosis of the renal vascular media. Multifocal perivascular and peribronchial lymphoid cell infiltrates were seen in the lungs of aged mice. Mild to moderate foci of periductulal infiltrates of lymphocytes were observed in portal triads of the liver in aged mice. Representative histological features of these inflammatory lesions observed at 24 months of age in B6 mice were shown in Fig. 1A, 1C, 1E, 1G and 1I. Diffusely infiltrating foci were not observed, except in the salivary gland. Many of the mice frequently had lesions in several organs. The number of mice with lesions in two, three, four or five organs was, at 12 months; two, three, three and two; at 18 months: seven, nine eight and six; and at 24 months: 12, 17, 14 and 11, respectively. In contrast, inflammatory lesions could not be recognized in other organs including thyroid, stomach, adrenal, ovary, testis or prostate.

Immunohistologic study of affected organs

To identify cell populations of the infiltrating mononuclear cells within the lesions in aged B6 mice, an ABC technique using monoclonal antibodies directed to cell surface antigens was employed. Serial frozen sections were stained with anti-Thy1.2, -L3T4,-Lyt2, and immunoglobulins. Most of these lymphocytes present in the lesions showed a positive reaction with monoclonal antibody to Thy1.2, L3T4 in all of the examined specimens, while a small number of lymphocytes reacted with monoclonal antibody to Lyt2 within the inflammatory lesions (Fig. 2). Although the proportion of L3T4$^+$ cells and Lyt2$^+$ was varying from organ to organ affected as shown in Table 2, predominance of Thy1.2$^+$ cells within these lesions was preserved in each organ. In addition, the proportion of the different phenotypes remains constant in each group of age and sex. In contrast, there appeared a small number of surface imunoglobulin-positive lymphocytes in these affected organs, which were seen sporadically in the inflammatory lesions (data not shown).

Detection of circulating autoantibody

As shown in Table 3, circulating autoantibodies of IgG type were detected by indirect IF test in the sera of aged B6 mice, and their incidence in each organ was gradually increased with advancing age in both sexes, in addition to the presence of antinuclear antibodies as reported previously (Kato & Hirokawa, 1989). Fig. 3 shows serum titre of autoantibodies and a correlation with the incidence of the each corresponding lesions in individual mice at 24 months of age. Circulating autoantibodies against epithelial duct cells of salivary gland (Fig. 1B) were detected in the sera of mice with sialadenitis. Antibodies against tubular epithelial cells of the kidney (Fig. 1D), islet cells of the pancreas (Fig. 1F), bronchiolar epithelial cells of the lung (Fig. 1H), and ductulal epithelial cells of the portal triads in liver (Fig. 1J) were detected in the sera of mice with lesions in each corresponding organ. Such autoantibodies were not detected in the sera of normal young B6 mice. In addition, circulating autoantibodies against other organs without cellular infiltration were detected in thymic epithelial cells and spermatocytes, as shown in Table 3.

DISCUSSION

Our results indicate that organ-specific autoimmune lesions develop spontaneously in non-autoimmune B6 SPF mice associated with ageing process. The affected organs were revealed to be localized in the submandibular salivary gland, kidney, pancreatic islet, lung and liver. These inflammatory lesions developing with ageing process can be considered to be autoimmune in nature, since circulating autoantibodies were detected in each corresponding organ in aged B6 mice respectively; anti-salivary duct antibody in sialadenitis; anti-tubular epithelial cell antibody in renal lesion; anti-islet cell antibody in pancreatic lesion; anti-bronchiolar epithelial cell antibody in pulmonary lesion; and anti-bile ductular antibody in cholangiolitis. Although it remains unknown why the lesions are restricted to these organs, it is possible that age-related disturbance of regulatory T cell functions normally responsible for immunological tolerance causes the expansion of self-reactive clones of T cells against tissue-specific autoantigens in these organs. In terms of the spectrum of organs affected, Sakaguchi & Sakaguchi (1989) have recently described that genetic factors play a role in determining the susceptibility to organ-specific autoimmune diseases.

It has been reported that spontaneous occurrence of lymphocytic infiltration in several organs similar to the organ-specific autoimmune diseases develop in various strains of autoimmune disease-prone mice. That is, autoimmune sialade-

Table 3. Search for circulating autoantibodies in sera from mice of various ages

Age (month)	No. of testing sera	No. of mice giving IF-positive reaction						
		Salivary gland	Kidney	Pancreatic islet	Lung	Liver	Thymus	Testis
3	10	0	0	0	0	0	0	0
6	10	1(0)	0	0	0	0	0	0
18	15	13(11)	9(7)	9(7)	10(7)	8(6)	3(0)	2(0)
24	20	19(18)	17(15)	18(16)	17(15)	16(14)	5(0)	4(0)

Testing serum was harvested from individual mice; sera were diluted 20-fold for testing autoantibodies. Numbers in parentheses indicate mice with both an autoantibody and an inflammatory lesion.

* Male mice were included: 8 of 15 at 18 months of age, and 10 of 20 at 24 months of age.

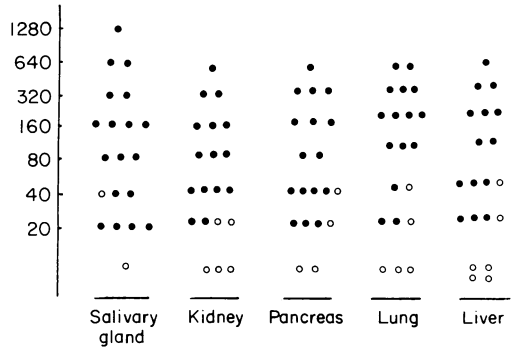

Fig. 3. Titre of autoantibodies assessed by indirect immunofluorescence from 24-month-old C57BL/6 mice. The serum titre of autoantibodies was determined at the maximum dilution giving positive reaction on frozen section. Autoimmune lesion histologically overt (●), or histologically in normal range (○).

nitis appeared spontaneously in NZB, NZB/W, SL/Ni, and MRL mice (Kessler, 1968; Takeda & Ishikawa, 1983; Hoffman *et al.*, 1984), which shows similarities to that of human Sjögren's syndrome. Spontaneous occurring sialadenitis has been also shown in the non-obese diabetic (NOD) mouse (Miyagawa *et al.*, 1986). Inflammatory vascular changes have been reported in the liver and kidney of MRL, and SL/Ni mice (Jabs & Prendergast, 1987). In particular, 75% of MRL mice have a polyarteritis involving medium-sized arteries of the kidney, genital organs and heart (Alexander *et al.*, 1985). In autoimmune liver disease, it has been reported that the bile antigen in bile may be derived from the salivary duct epithelium by the immunodiffusion studies, suggesting that there is a cross-reaction between salivary gland materials and bile duct epithelial cells (Mcfarlane *et al.*, 1976). The spontaneous occurrence of pulmonary vasculitis has been reported in NZB/W mice,, especially in aged mice (Staszak & Harbeck, 1985; Harbeck, Launder & Staszak, 1986).

We have recently reported the spontaneous occurrence in aged BDF1 mice of an autoimmune sialadenitis which show similarities to that of Sjögren's syndrome in humans (Hayashin *et al.*, 1988). It was interesting to note that spontaneously occurring organ-specific autoimmune lesions developing with advance of age could be observed in not only the salivary gland of F1 hybrids (BDF1), but also the multiple organs of maternal strain. The infiltrating cells within the lesions in the salivary glands in aged BDF1 mice were mainly composed of Thy1.2$^+$, L3T4$^+$ cells. The present results also confirmed that the tissue-infiltrating, autoreactive T cells in the multiple organs in aged mice have the major phenotype of L3T4 (from 70 to 80%). Recently, Jabs & Prendergast (1987) have found that the majority of infiltrating lymphocytes in the lacrimal gland inflammatory lesions and renal vasculitis of MRL mice expressed L3T4. Moreover, Koike *et al.* (1987) have reported that most of the infiltrating cells in pancreatic islets of NOD mice were L3T4$^+$ lymphocyte, and that *in vivo* administration of anti-L3T4 monoclonal antibody has been effective in preventing spontaneous diabetes in NOD mice. The infiltrating lymphocytes responsible for the pulmonary lesions in NZB/W mice have also found to express L3T4 phenotype (Harbeck *et al.*, 1986). From these data, it can be postulated that L3T4$^+$ cells would play a central role in the induction of organ-specific autoimmune diseases. However, recent studies demonstrate that the induction of diabetes in the adoptive transfer system of NOD mice is dependent on both the L3T4$^+$ and Lyt2$^+$ T cells (Bendelac *et al.*, 1987; Miller *et al.*, 1988). Thus, more research is necessary and is, indeed, in progress, to obtain directly various clones of autoreactive T cells affecting various organs.

In the B6 mice we used, we have found apparent T cell-dependent immunologic disorders with advancing age, as reported previously (Hirokawa *et al.*, 1984). It has been shown that the percentage of Lyt2$^+$ cells in the spleen decreased with advancing age, whereas that of L3T4$^+$ cells was relatively constant throughout the life in B6 mice. Consequently, splenic T cell subpopulations showed an apparent proportional imbalance at 24-month-old of B6 mice (Utsuyama & Hirokawa, 1987). Moreover, various serum levels of autoantibodies apparently increase with advance of age in B6 mice (Kato & Hirokawa, 1989). Since cell fusion technique revealed that young mice had numerous B cell clones potential to produce autoantibodies reactive to wide variety of self antigen, it was strongly suggested that the enhanced autoantibody production in aged mice is due to the age-associated degradiation of regulatory system suppressing autoantibody production (Kato & Hirokawa, 1989). It is possible that multiple organ-specific autoimmune lesions in aged B6 mice could develop as a consequence of age-associated disturvance of regulatory T cell functions. Recently, Taguchi *et al.* (1986) have reported that

experimentally induced, multiple, organ-localized autoimmune diseases in thyroid, salivary gland, stomach, prostate, ovary, and testis in rat thymus-grafted BALB/c nu/nu mice. However, spontaneously occurring, multiple, organ-specific autoimmune lesions in non-autoimmune prone mice have not yet been reported until this strain of aged mice, which may become an appropriate animal model for both the pathogenesis of organ-specific autoimmune diseases and the autoimmune mechanism associated with the ageing process in humans.

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