Induction of Experimental Allergic Sialadenitis in Mice

YOSHIO HAYASHI, DDS, D Med Sci,
MITSUNOBU SATO, DDS, PhD, and
KATSUIKU HIROKAWA, MD, D Med Sci

From the Departments of Laboratory Medicine and of Oral and Maxillofacial Surgery, Tokushima University School of Dentistry, Tokushima, Japan, and the Department of Pathology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

This article reports that sialadenitis developed in female CRJ:CD-1 mice thymectomized 3 days after birth and later immunized with a homogenate of the submandibular salivary gland emulsified with complete Freund's adjuvant. Significant inflammatory changes did not develop in various control groups, including animals thymectomized at Day 3 but not immunized and animals not thymectomized on the day of birth but immunized. Because a more marked decrease of Lyt 2$^+$ cells was found in mice thymectomized on Day 3 after birth than in neonatally thymectomized mice, thymectomy at 3 days of age is more effective for the induction of sialadenitis, presumably by markedly decreasing a population of suppressor T cells. The lesions observed in mice with sialadenitis were mostly composed of small and medium-sized lymphocytes stained by anti-Thy 1.2 and Lyt 2 antibodies and in later stages by immunoglobulin-containing cells in the periphery of inflammatory lesions. (Am J Pathol 1985, 118:476–483)

SEVERAL experimental animal models in guinea pig, rat, rabbit, and mouse, which resemble Sjögren's syndrome and other chronic sialadenitis in man have been reported and studied for histopathologic and immunologic features.[1-13] Accumulated evidence strongly suggests that these diseases in man are a consequence of dysfunction of some loops of the immune systems within the host.[14-18] Therefore, these animal models provide systems suitable for study of the pathogenesis of certain autoimmune diseases affecting the salivary glands. However, repeated attempts to produce destructive and long-lasting lesions of the salivary glands have met with little success. In addition, the significance of the pathogenetic role of the immune system, including cell-mediated and humoral immunity, in the induction of experimental sialadenitis is not yet understood, although some investigators have stressed the importance of humoral immunity in the pathogenesis of experimental sialadenitis.[6,7]

While it is well known that neonatal thymectomy can induce severe impairment of cell-mediated immunity,[19,20] neonatal thymectomy of certain mouse strains that are susceptible to autoallergic disease was reported to cause acceleration of the development of the disease.[21] Moreover, even in some mouse strains that are genetically not susceptible to autoallergic disease, it was reported that neonatal thymectomy induced disorders resembling autoimmune diseases in man in the thyroid, ovary, testis, and kidney.[22-27] A possible mechanism for an increase in the occurrence of allergic disorders in neonatally thymectomized animals is deficiency in T-cell-mediated immunity.

In this communication, we report an induction of allergic sialadenitis in mice by perinatal thymectomy followed by sensitization with a submandibular-gland homogenate emulsified with complete Freund's adjuvant, and we discuss herein the possible immune mechanism.

Materials and Methods

Animals

Female CRJ:CD-1 mice were purchased from a closed colony of Charles River Japan, Inc. (Atsugi, Japan) and used in the present study. The animals were acclimatized to a daily cycle of alternating 12-hour periods of light and darkness in an air-conditioned room at 24 ±

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Address reprint requests to Dr. Y. Hayashi, Department of Laboratory Medicine, Tokushima University School of Dentistry, 3 Kuramoto-cho, Tokushima 770, Japan.

1.1 C throughout the experimental period. They were given food and water *ad libitum*.

Experimental Design

Thymectomy was performed on Day 0 or Day 3 after birth. A homogenate emulsion of the submandibular salivary gland from female CRJ:CD-1 mice 8–10 weeks of age was prepared by chopping and homogenizing the tissue in an equal volume (wt/vol) of phosphate-buffered saline (PBS, pH 7.2) and then emulsifying the homogenate with an equal volume (vol/vol) of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich). This emulsion was injected subcutaneously into the four footpads of mice.

A total of 180 mice used for the present study were divided into five groups as follows. Group I consisted of 65 mice that were thymectomized 3 days after birth. At 28 and 42 days of age, these animals were sensitized with a homogenate of the submandibular salivary gland in CFA. They were sacrificed by cervical dislocation sequentially during various time intervals ranging from 1 to 16 weeks after the last sensitization. Group II consisted of 52 nonthymectomized mice that were sensitized with a homogenate of the submandibular salivary gland in CFA at 28 and 42 days of age. Thereafter, these animals were processed in the same manner as described for Group I. Group III consisted of 15 mice that were thymectomized 3 days after birth. PBS alone was injected subcutaneously into the footpads of these animals at 28 and 42 days of age. Then they were sacrificed 1, 3, and 5 weeks after the last injection of PBS. For Group IV, PBS emulsified with CFA was injected into the footpads of 15 nonthymectomized mice at 28 and 42 days of age. These animals were sacrificed 1, 3, and 5 weeks after the last injection. Group V consisted of 33 mice thymectomized immediately after birth. These animals were sensitized with a homogenate of the submandibular salivary gland in CFA at 28 and 42 days of age and were sacrificed 1, 3, 5, and 16 weeks after the last sensitization.

All organs were removed from the sacrificed mice, fixed with 4% phosphate-buffered formaldehyde (pH 7.2) and prepared for histologic examination. The sections were stained with hematoxylin and eosin (H&E), by the periodic acid–Schiff method or with elastic–van Gieson stain. Grading of the inflammatory lesions of submandibular salivary glands were classified according to the method proposed by White and Casarett.[11] Namely, longitudinal sections of all glands were examined under × 150 magnification and scored for degree of the infiltration of mononuclear cells, including lymphocytes, plasma cells, and macrophages, as follows: 1 indicates that 1 to 5 foci being composed of more than 20 mononuclear cells per focus were seen, 2 indicates that more than 5 such foci were seen but without significant parenchymal destruction, 3 indicates that multiple confluent foci were seen in moderate degeneration of parenchymal tissue, and 4 indicates extensive infiltration of the glands with mononuclear cells and extensive parenchymal destruction.

Fluorescent Antibody (FA) Staining Technique

Frozen sections approximately 4 μ in thickness were prepared from the submandibular salivary glands and stained by an indirect immunofluorescent antibody technique. Immediately before staining, the sections were rinsed in cold PBS and fixed in 95% ethanol at 4 C for 5 minutes and then dried. The fixed preparations were incubated at 37 C for 1 hour with each of rat monoclonal antibodies to Thy 1.2, Lyt 1, and Lyt 2 (Becton-Dickinson, Inc., Sunnyvale, Calif). They were then washed with cold PBS for 30 minutes by mechanical shaking and stained with fluorescein-conjugated anti-rat IgG rabbit globulin (Miles Laboratories, Elkhart, Ind) at 37 C for 1 hour. After being washed, they were mounted with buffered glycerol (pH 9.5) and examined under a Nikon fluorescence microscope (Nikon Co., Tokyo, Japan). Controls were carried out with rat whole serum (Cappel Laboratories, Cochranville, Pa).

Indirect Peroxidase-Labeled Antibody Method

The paraffin-embedded specimens were cut into serial sections approximately 4 μ in thickness. The paraffin sections were deparaffinized with xylene and rehydrated step by step with descending concentrations of ethanol. These preparations were washed three times with PBS and incubated in PBS containing 0.25% trypsin at 37 C for 1 hour. After being washed three times with PBS, they were incubated with an appropriate dilution of rabbit serum to each of mouse IgG, IgA, or IgM (Miles Laboratories) for 30 minutes at room temperature and then by a rinse with PBS for 30 minutes. Thereafter, anti-rabbit IgG labeled with horseradish peroxidase (Miles Laboratories) was applied in a dilution of 1:40 in PBS for 30 minutes at room temperature. After being washed three times with PBS, the peroxidase was localized by treatment of the samples 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 minutes and after being washed with water, these samples were counterstained with 1% methyl green for 1 hour. In the experiments, anti-mouse IgG, IgA, and IgM sera were used at a dilution of 1:20.

Detection of Antisalivary Duct Antibody

Sera were harvested from mice sensitized with a homogenate of the submandibular salivary gland and examined by the FA staining technique for the presence of antisalivary duct antibody.

Frozen sections 4 μ in thickness of submandibular salivary gland from the untreated female CRJ:CD-1 mice were prepared and reacted with a testing serum and fluorescein-conjugated rabbit antiserum to mouse IgG (Miles Laboratories) in the same manner as described for the FA staining technique. The titer of antisalivary duct antibody was expressed as the highest dilution of testing serum giving positive staining on the frozen sections. To confirm the specificity of the FA reaction, absorption tests were carried out as follows. The submandibular salivary gland, liver, kidney, and adrenal were harvested from the untreated mice and chopped with scissors. Then they were homogenized gently in an equal volume (wt/vol) of cold PBS and centrifuged at 2000 *g* for 30 minutes. The resulting pellets were suspended in an appropriate volume (vol/vol) of 20-fold dilution of a testing serum and stood for 1 hour at 37 C. Thereafter, this mixture was centrifuged at 5000 *g* for 20 minutes, and the supernatant was used as a testing sample for FA staining. In addition, tissue sections from pancreas, thyroid, and kidney of the treated mice were examined by the FA staining technique for immunoreactivity of antisalivary duct antibody.

Analysis of Surface Markers on Spleen Cells

The surface markers defined by monoclonal antibodies to Thy 1.2, Lyt 1, and Lyt 2 on the spleen-cell subpopulation were analyzed by immunofluorescence assay.

Table 1—Degree of Inflammatory Infiltrate in Allergic Sialadenitis in Mice

Weeks after last immunization	Number of mice	Number of mice with lesion (Grades 0–4)*					Mean grade of lesion ± SD	% Incidence†
		0	1	2	3	4		
Group I								
1	5	3	1	0	0	1	1.0 ± 1.5	20.0
2	12	2	5	3	2	0	1.4 ± 1.1‡	41.7
3	10	1	3	3	2	1	1.9 ± 1.1	60.0
4	10	2	2	3	2	1	1.8 ± 1.0	60.0
5	11	2	4	3	1	1	1.6 ± 1.2	45.5
8	7	1	0	3	3	0	2.1 ± 1.0	85.7
16	10	2	0	2	3	3	2.5 ± 1.4‡	80.0
Group II								
1	5	4	1	0	0	0	0.2 ± 0.4	
2	12	8	4	0	0	0	0.3 ± 0.5	
3	10	7	3	0	0	0	0.3 ± 0.5	
4	10	7	3	0	0	0	0.3 ± 0.5	
5	5	4	1	0	0	0	0.2 ± 0.4	
8	5	4	1	0	0	0	0.2 ± 0.4	
16	5	4	1	0	0	0	0.2 ± 0.4	
Group III								
1	5	4	1	0	0	0	0.2 ± 0.4	
3	5	5	0	0	0	0	0	
5	5	5	0	0	0	0	0	
Group IV								
1	5	5	0	0	0	0	0	
3	5	5	0	0	0	0	0	
5	5	5	0	0	0	0	0	
Group V								
1	7	5	2	0	0	0	0.3 ± 0.5	
3	10	7	3	0	0	0	0.3 ± 0.5	
5	10	8	2	0	0	0	0.2 ± 0.4	
16	6	3	3	0	0	0	0.5 ± 0.5	

* Inflammatory lesions were divided into four grades according to the method of White and Casarett.[11]

† Percent incidence indicates the proportion of numbers of mice with inflammatory lesions of more than Grade 2 versus the total number of mice examined.

‡ The difference between the values indicated was statistically significant ($P < 0.05$) (Mann–Whitney U test).

Experimental groups were divided into five groups as follows: Group I: thymectomized at Day 3 after birth and later immunized with a homogenate of submandibular salivary gland in CFA; Group II: not thymectomized but immunized; Group III: thymectomized at Day 3 after birth but not immunized; Group IV: neither thymectomized nor immunized, but given CFA; Group V: thymectomized at Day 0 after birth and later immunized. See the text for the details of the immunization schedule.

Spleen-cell suspensions from mice were prepared in RPMI-1640 according to the method of Schooley.[28] Briefly, spleens were given injected RPMI-1640, teased gently with a forceps and a needle, and dispersed by repeated flushing with a 5-ml syringe through a 22-gauge needle. The dispersed cells were washed once with RPMI-1640 and then resuspended to an appropriate concentration. 5×10^6 cells in 0.1 ml were mixed with 0.1 ml of each of rat monoclonal antibodies to Thy 1.2, Lyt 1, and Lyt 2 (Becton-Dickinson), and incubated at room temperature for 30 minutes. The cells were then centrifuged and washed three times with Tris–PBS (pH 7.4), resuspended in 0.1 ml of the fluorescein-labeled goat anti-rat IgG (Miles Laboratories), and incubated at room temperature for 15 minutes. The cells were washed three times with the same buffer, resuspended in a drop of 7% bovine serum albumin (Sigma Chemical Co., Saint Louis, Mo) in Tris-PBS and smeared onto glass slides. The smears were air-dried, fixed for 5 minutes in 95% ethanol, mounted with glass coverslips in a drop of buffered glycerine (pH 9.5), and examined under a fluorescence microscope. For quantitation of positive staining cells, more than 300 spleen cells were counted for each sample.

Mitogenic Response

Mouse spleen cells (5×10^5) were incubated with an optimal concentration of either phytohemagglutinin (PHA, 1 μg/ml, Wellcome Reagents, Greenville, NC), concanavalin A (Con A, 1 μg/ml, Sigma Chemical Co., St. Louis, Mo) or E. coli lipopolysaccharide (LPS; 1 μg/ml, Difco Laboratories, Detroit, Mich) in 200 μl of RPMI supplemented with 10% fetal calf serum for 76 hours in a humidified atmosphere of 5% CO_2 in air. Thereafter, 1 μCi of [^{3}H]-thymidine (specific activity, 20 Ci/mmol; Radiochemical Center, Amersham, England) was added to each well for 6 hours, the cells were harvested, and the incorporation of thymidine into DNA was measured in triplicate wells with a liquid scintillation counter.

Electron-Microscopic Observations

For transmission electron microscopy, the tissues were fixed for 2 hours in 4% glutaraldehyde buffered with 0.1 M phosphate (pH 7.3) containing 0.005 M calcium chloride and then dissected into small pieces of about 2 cu mm. After being washed in the phosphate buffer, these materials were postfixed with 2% osmium tetroxide for 1 hour, dehydrated step by step with ethanol, and embedded in Epon 812. One-micron sections were stained with 1% toluidine blue and examined under a light microscope. After the appropriate fields were selected, ultrathin sections were cut on a LKB ultramicrotome and stained with uranyl acetate and lead hydroxide. The finished preparations were observed under a Hitachi electron microscope, Model H-500.

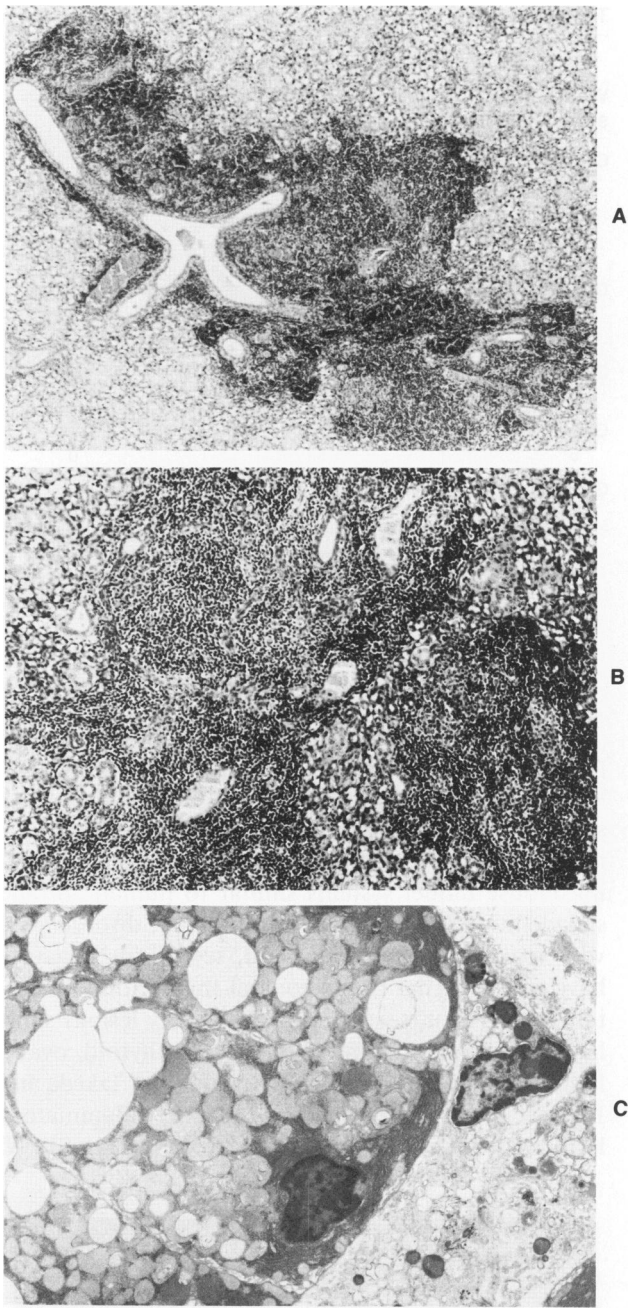

Figure 1—Histologic appearance and electron micrograph of allergic sialadenitis in mice. **A** and **B**—Photomicrographs demonstrating a severe destructive lesion (Grade 4) of the submandibular salivary gland with extensive infiltration of mononuclear cells. Infiltrative mononuclear cells consist of small and medium-sized lymphocytes. **C**—Electron micrograph revealing damage sustained by acinar epithelial cells. (**A**, H&E, ×70; **B**, H&E, ×200; **C**, ×3000.)

Table 2—Search for Anti-Salivary Duct Antibody in Sera From Mice of Each Experimental Group

Weeks after sensitization	Number of testing sera*	Number of mice giving FA-positive reaction (antibody titer†)				
		×5	×10	×20	×80	×160
Group I						
2	4	0	0	0	0	0
4	6	1	0	0	0	0
8	5	2	1	1	0	0
16	5	0	2	1	1	1
Group II						
8	5	0	0	0	0	0
16	5	0	0	0	0	0
Group III						
5	5	0	0	0	0	0
Group IV						
5	5	0	0	0	0	0
Group V						
16	5	0	0	0	0	0

* Each testing serum was harvested from individual mice.
† The titer of anti-salivary duct antibody is expressed as the highest serum dilution giving an FA-positive reaction.
Titers of anti-salivary duct antibody in all sera obtained from individual mice immediately prior to immunization were <×5.

Results and Discussion

Although it has been reported that sialadenitis appeared spontaneously in NZB /NZW and SL/Ni mice with lesions resembling human autoimmune diseases such as systemic lupus erythematosus and polyarteritis nodosa,[4,29-32] studies establishing and characterizing mouse model systems similar to human chronic sialadenitis with associated immunologic disorders are very sparse. On the other hand, several investigators have reported that only neonatal thymectomy in certain strains of mice causes inflammatory lesions similar to human autoimmune diseases in thyroid, ovary, kidney, testis, and stomach.[22-26] In addition, Takeda and Ishikawa[4] have recently reported that the inflammatory lesion of the submandibular salivary gland in SL/Ni mice is markedly aggravated by immunization with a homogenate of the submandibular salivary gland. Thus, for the induction of inflammatory lesions in the submandibular salivary gland of female CRJ:CD-1 mice, the mice were thymectomized and later immunized with a homogenate of the submandibular salivary gland. Table 1 summarized the degree of mononuclear cell infiltration and parenchymal destruction in individual mice of each experimental group. Allergic sialadenitis was induced exclusively in Group I mice, which had been thymectomized 3 days after birth and then sensitized with a homogenate of the submandibular salivary gland in CFA. Generally, inflammatory lesions could not be observed in any of the mice of any group in organs such as pancreas, thyroid, kidney, and spleen but were seen only in the submandibular and parotid salivary glands. However, the number of inflammatory lesions of the parotid gland was markedly decreased, as compared with the submandibular salivary gland; and 16 weeks after the last immunization, an inflammatory lesion of Grade 1 was observed only in 6 mice of Group I. Also, in Group I mice inflammatory lesions developed in the submandibular salivary gland more rapidly than in other groups. It is interesting to note that grading of the inflammatory lesion and the percentage of incidence which is proposed as an inflammatory lesion of more than Grade 2 are increased only in Group I mice according to time intervals after the last sensitization. In particular, an inflammatory lesion of Grade 4, indicating extensive infiltration of mononuclear cells and destruction of parenchymal tissue, was seen in 3 of 10 mice examined (Figure 1). Moreover, as shown in Table 2, anti-salivary duct antibody was detected only

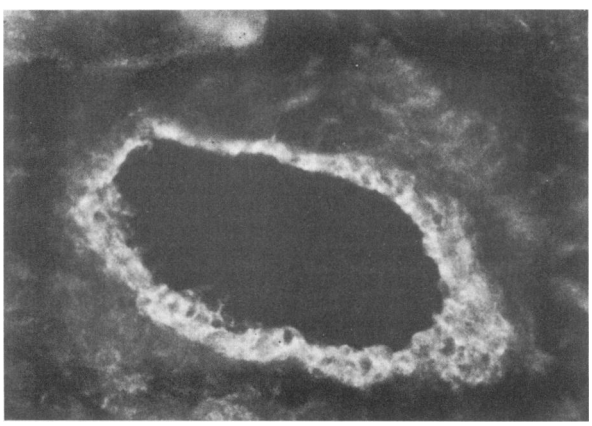

Figure 2—Anti-salivary duct antibody detected in the serum from certain Group I mice 16 weeks after the last immunization. The dilution of testing serum used was 80-fold.

Table 3—Changes of Spleen Cell Subpopulation and Its Mitogenic Responsiveness in Mice Thymecomized at Different Times After Birth

Thymectomized at (n = 5)	T-cell subpopulations (%)*				Mitogenic response (cpm)*		
	Thy 1.2	Lyt 1	Lyt 2	Lyt 1/Lyt 2	PHA	Con A	LPS
Day 0 after birth	11.3 ± 0.9[a]	19.7 ± 2.7[g]	6.3 ± 0.9	5.4 ± 2.2	3206 ± 354[c]	3618 ± 787[d]	7536 ± 1610
Day 3 after bith	18.0 ± 0.8[e]	26.6 ± 1.5	5.4 ± 1.3[f]	6.6 ± 2.2	1314 ± 381[g]	2103 ± 516[h]	8766 ± 1400
Control (nonthymectomized)	32.8 ± 1.7[i]	34.0 ± 3.4[j]	7.3 ± 0.9[k]	4.8 ± 0.5	23630 ± 2547[l]	40199 ± 1835[m]	7759 ± 774

* See text for details.

Statistical differences: a < i, $P < 0.01$; e < i, $P < 0.01$; b < j, $P < 0.01$; f < k, $P < 0.05$; c < l, $P < 0.01$; g < l, $P < 0.01$; d < m, $P < 0.01$; h < m, $P < 0.01$.

For examination of the effect of thymectomy on female CRJ: CD-1 mice, each of 5 animals thymectomized at day 0 or day 3 after birth but not immunized was tested for the spleen cell subpopulation and its mitogenic responsiveness 6 weeks after birth.

in some Group I mice. Figure 2 shows a representative result of FA-positive staining. When the sera containing anti-salivary duct antibody were absorbed by a homogenate of submandibular salivary gland from the untreated mice, this antibody activity was completely eliminated; whereas the sera were not affected by the homogenates of the other organs such as liver, kidney, and adrenal. In addition, tissues such as pancreas, thyroid, and kidney showed FA-negative reaction with anti-salivary duct antibody. On the other hand, Group V mice which had been neonatally thymectomized and then sensitized with a homogenate of submandibular salivary gland in CFA neither developed inflammatory lesions of more than Grade 2 nor raised anti-salivary gland duct antibody.

Thus, we examined the effect on the host immune function of thymectomy, which was performed at various time intervals after birth. As shown in Table 3, more significant decreases were found in the percent populations of Thy 1.2^+ cells ($P < 0.01$) and Lyt 1^+ cells ($P < 0.01$) in spleen cells of neonatally thymectomized mice and in those of Thy 1.2^+ cells ($P < 0.01$) and Lyt 2^+ cells ($P < 0.05$) in mice thymectomized 3 days after birth than in those of nonthymectomized mice. In particular, a more marked decrease of Lyt 2^+ cells was found in mice thymectomized 3 days after birth than neonatally thymectomized mice. This may indicate that thymectomy at 3 days after birth is effective for an induction of allergic sialadenitis, presumably by markedly decreasing a population of suppressor T cells. In addition, PHA or Con A responsiveness of spleen cells from mice thymectomized 3 days after birth was markedly decreased as compared with that of neonatally thymectomized mice. Leventhal et al[14] already reported that a decrease in cell-mediated immunity as assessed by mitogenic response of peripheral blood to phytohemagglutinin and skin testing to dinitrochlorobenzene was observed in the patients with Sjögren's syndrome. Also, Keyes et al[33] described in NZB/NZW mice an age-related decline in responsiveness of spleen cells to various mitogens such as PHA, Con A, and lipopolysaccharide associated with an increase in the onset of a disease resembling Sjögren's syndrome in man and the progression of lymphoid cell infiltration into lacrimal and salivary glands.

The mononuclear cells that infiltrated chronic sialadenitis induced by immunization with a homoge-

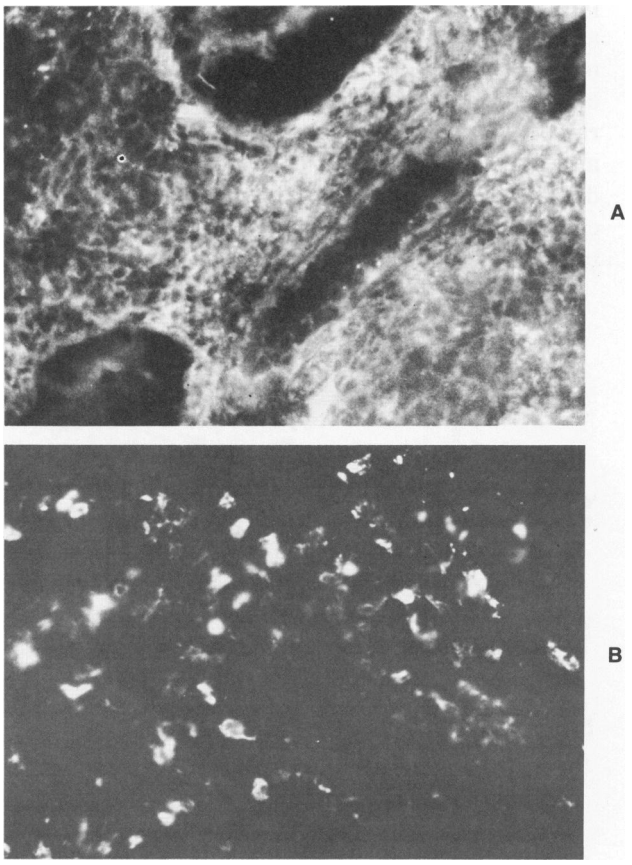

Figure 3—Analysis of surface markers of infiltrative lymphocytes in allergic sialadenitis. Subpopulations of infiltrative lymphocytes in lesions were analyzed by the indirect immunofluorescent staining technique with the use of monoclonal antibodies. Thy 1.2+ (**A**) and Lyt 2+ (**B**) lymphocytes were observed in the inflammatory focus.

Table 4—Analysis of Subpopulations of Ig-Bearing Cells in Inflammatory Lesions

Ig class	Percentage	Count/focus
IgG	53.7 ± 8.9	24 ± 7
IgA	27.0 ± 7.9	13 ± 8
IgM	15.2 ± 4.9	7 ± 4

Infiltrative mononuclear cells forming an inflammatory focus were examined by the indirect peroxidase-labeled antibody method for cells carrying each class of Ig. "Percentage" means the proportion of the numbers of cells carrying each class of Ig versus absolute counts of Ig-bearing cells per focus.

nate of the submandibular salivary gland consisted mainly of small and medium-sized lymphocytes, although a small number of plasmacytoid cells appeared in the inflammatory lesions observed at 8 and 16 weeks after the last sensitization. These mononuclear cells present in the lesions showed a positive reaction with anti-Thy 1.2 and anti-Lyt 2 antisera in all of the examined specimens (Figure 3). Moreover, Ig-bearing cells were demonstrated only in the specimens obtained at 8 and 16 weeks after the last sensitization (Table 4). IgG-positive cells were especially predominant in the lesions, although there was no deposit of Ig in any parenchymal portion of the submandibular salivary gland affected. These Ig-bearing cells were located in the periphery of the inflammatory lesions (Figure 4). The above findings suggest that in addition to the tissue damage through Lyt 2-positive lymphocytes defined as cytotoxic/suppressor cells by anti-Lyt 2 antiserum, the mechanism of antibody-dependent cell-mediated cytotoxicity can also play a major role in the formation of the inflammatory lesion. Also, Paget et al[34] reported that in experimental autoimmune thyroiditis in guinea pigs, T-lymphocytes were the predominant infiltrating lymphocytes and B-lymphocytes constituted most of the remainder. In addition, they showed that the infiltrates of animals with thyroiditis contained cells capable of mediating antibody-dependent lymphoid cell-mediated cytotoxicity. Further investigation of the role and significance of inflammatory-cell infiltration in the formation of experimental allergic sialadenitis in mice are now in progress in our laboratories.

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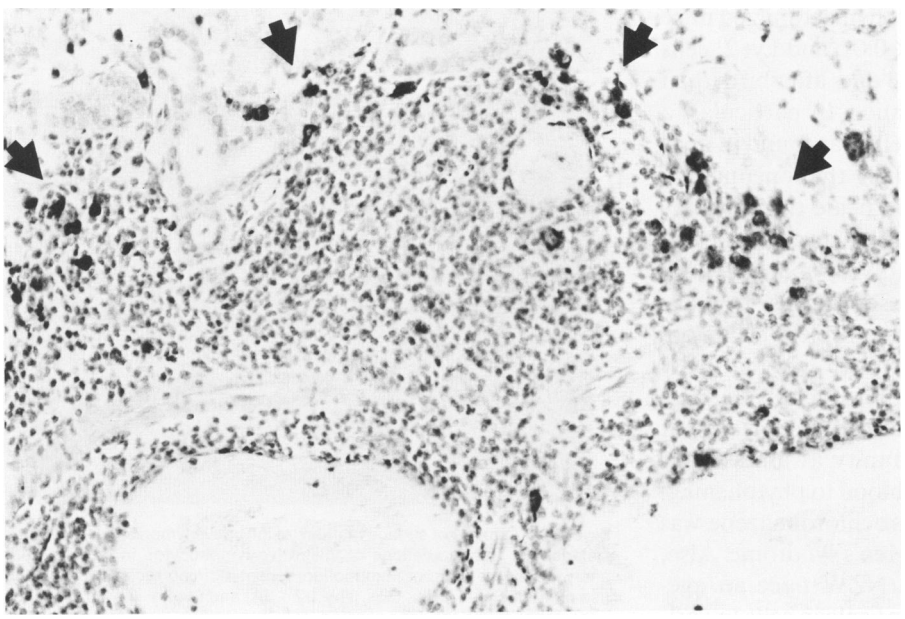

Figure 4—Detection of IgG-bearing plasma cells in infiltrative mononuclear cells in allergic sialadenitis by the indirect peroxidase-labeled antibody method. IgG-bearing plasma cells (*arrows*) appear in later stages during the course of formation of allergic sialadenitis in the periphery of the inflammatory lesions.

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