The Presence of Cytotoxic Autoantibody to Lacrimal Gland Cells in NZB/W Mice

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New Zealand Black and White F₁ hybrid mice (NZB/W mice) spontaneously develop an autoimmune disease which provides us with a suitable animal model for Sjögren's syndrome. With increasing age, these mice develop foci of mononuclear cell infiltration in the lacrimal and salivary glands, which closely resemble the lesions seen in patients with Sjögren's syndrome. We studied the cell-mediated and antibody-mediated immune responses of NZB/W mice to lacrimal gland cells. Lacrimal gland acinar cells were isolated from 2-month-old NZB/W or BALB/c mice for the target of ⁵¹Cr-release assay. There was no statistically significant difference in the spleen cell-mediated cytotoxicity to lacrimal gland cells among NZB/W mice of different ages (2, 5, and 8 months old). With increasing age, on the other hand, we found a statistically significant increase in the titers of autoantibodies to lacrimal gland cells in NZB/W mice, while aged BALB/c mice did not develop such antibodies. Fractionation of pooled positive sera by gel filtration revealed that this cytotoxic activity was mostly recovered in the IgM fraction. The tissue absorption study showed that these antibodies cross-reacted with salivary gland and kidney. Invest Ophthalmol Vis Sci 26:214-219, 1985

Sjögren's syndrome is a chronic, relatively common autoimmune disorder characterized by the mononuclear cell infiltration of the lacrimal and salivary glands. This infiltrative process causes the progressive destruction of exocrine glands and eventually brings in intractable symptoms of keratoconjunctivitis sicca and xerostomia.¹ ² ³ The etiology of Sjögren's syndrome is still unknown, however it has been suggested that autoimmune mechanisms are involved in the pathogenesis of this disease.

New Zealand Black/White F₁ hybrid mice (NZB/W mice) have been found to develop spontaneously an autoimmune disease with aging.⁴ ⁵ They display a variety of autoantibodies and usually die from severe renal failure, which has been caused by immune complex-mediated glomerulonephritis.⁶ ⁷ Kessler et al showed that aged NZB/W mice exhibited a characteristic mononuclear cell infiltration in the lacrimal gland.⁸ ⁹ Recently, DeLuise et al, in a chronicologic study, investigated the histopathology of the lacrimal gland and showed that the changes started as early as 4 months of age.¹⁰ However, the exact etiology of the infiltration to these exocrine glands remains unknown. In terms of tissue injury, one possible hypothesis may be that the lacrimal gland cells could be the target of the host immune system. This study, using ⁵¹Cr-release assay, was undertaken to investigate whether there was any autoimmune response to lacrimal gland cells in NZB/W mice.

Materials and Methods

Animals

Female, 2-3-month-old NZB/W mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Also, 2- and 6-month-old female BALB/c mice were obtained from the Simonsen Laboratory (Gilroy, CA). Most of the NZB/W mice and 2-month-old BALB/c mice were used for the source of target cells, but some of the NZB/W mice were housed in the vivarium for a desired period. About 30% of the NZB/W mice died before reaching 8 months of age. Studies using these animals were performed in conformance with the ARVO Resolution on the Use of Animals in Research.

Chemicals and Reagents

Collagenase (type 2; 3.4.24.3 156 U/mg), hyaluronidase (HSEP; 3.2.1.35 9134 U/mg), and soybean trypsin inhibitor were purchased from Worthington Biochemical Inc. (Freehold, NJ). Ethylenediamine tetra-acetic acid (EDTA), carbamylcholine chloride,
and saponin were obtained from Sigma Chemicals (St. Louis, MO). Guinea pig complement was purchased from Gibco Laboratory (Grand Island, NY).

Culture Medium

Ham’s F-12 medium (F-12), heat-inactivated fetal calf serum (HIFCS), and Ca++, Mg+-free, Hanks' balanced salt solution (HBSS), were supplied by the Cell Culture Facility of University of California (San Francisco, CA). The isolation medium contained 0.1 mg/ml of soybean trypsin inhibitor and the cytotoxicity medium was supplemented additionally with 10% HIFCS and 10^-6 M of carbamylcholine as a secretagogue.

Isolation of Lacrimal Gland Cells

Lacrimal gland cells were isolated from either 2-month-old NZB/W or BALB/c mice, based on the method of Oliver.11 Each animal was killed by cervical dislocation and was perfused immediately with 10 ml of F-12 through the left ventricle in order to flush out the blood from the lacrimal gland. Following perfusion, both exorbital lacrimal glands were excised and rinsed in the isolation medium. After several cycles of washing, the glands were minced into fragments and rinsed in HBSS for 5 min. Fragmented glands then were transferred into Erlenmeyer’s flask (25 ml) with 5 ml of HBSS containing 0.76 mg/ml of EDTA, and the chelation was continued for 15 min at 37°C with continuous stirring. Chelated tissues were rinsed in the isolation medium for 5 min and transferred into another flask with 5 ml of enzyme solution. The enzyme solution consisted of F-12 medium containing 195 U/ml of collagenase and 300 U/ml of hyaluronidase. After the digestion for 15 min at 37°C, the tissues again were rinsed in HBSS for 5 min for the next chelation. This chelation-digestion cycle was repeated until the lacrimal gland dislocation and was perfused immediately with 10 ml of F-12 through the left ventricle in order to flush out the blood from the lacrimal gland. Following perfusion, both exorbital lacrimal glands were excised and rinsed in the isolation medium. After several cycles of washing, the glands were minced into fragments and rinsed in HBSS for 5 min. Fragmented glands then were transferred into Erlenmeyer’s flask (25 ml) with 5 ml of HBSS containing 0.76 mg/ml of EDTA, and the chelation was continued for 15 min at 37°C with continuous stirring. Chelated tissues were rinsed in the isolation medium for 5 min and transferred into another flask with 5 ml of enzyme solution. The enzyme solution consisted of F-12 medium containing 195 U/ml of collagenase and 300 U/ml of hyaluronidase. After the digestion for 15 min at 37°C, the tissues again were rinsed in HBSS for 5 min for the next chelation. This chelation-digestion cycle was repeated until the lacrimal gland tissues were dispersed considerably. The dispersed cells were washed twice with the isolation medium supplemented with 10% HIFCS and were forced gently through 500 µm and 25 µm Nytex screens (Tekto Inc.; Elsford, NY) for further dispersion. The cells were lightly centrifuged at 50 g for 5 min and suspended in the cytotoxicity medium for labeling. A part of the cell suspension was cytocentrifuged and stained with Diff-Quik (Dade Diagnostics, Aguada Puerto Rico, 00602) to check the population of the isolated cells.

Preparation of Target Cells

The isolated lacrimal gland cells were immediately labelled with 0.1 mCi of 51Cr (sodium chromate: specific activity 250–500 mCi/mg, Amersham; Arlington, IL) for 1 hr at 37°C. The labelled cells then were washed three times with a large volume of the cytotoxicity medium and suspended in this medium for 2 hr of preincubation. The lacrimal cells finally were suspended in the cytotoxicity medium at the concentration of 1 X 10^5 cells/ml. The viability of the target cells after the labelling usually was around 80%.

Spleen Cell-Mediated Cytotoxicity

The animals examined were composed of four groups: 2-, 5-, and 8-month-old NZB/W mice and 6-month-old BALB/c mice. They were killed by cervical dislocation and their spleens were excised aseptically. Minced spleens were wrapped up in a cotton gauze and squeezed in F-12 medium. After lysing red blood cells by hypotonic treatment, spleen cells were washed twice and suspended in the cytotoxicity medium at the concentration of 1 X 10^7 cells/ml. The viability of spleen cells was almost 100% as determined by trypsin blue dye exclusion test. One-tenth ml of target cell suspension prepared as described above, (10^6 cells/well) and 0.1 ml of effector cell suspension (10^6 cells/ml) were mixed at the effector to target cell ratio of 100/1 in the wells of microtitration plate. After incubation for 4 hr at 37°C, the plate was centrifuged to spin down the cells, and 0.1 ml of the supernatant medium was collected and counted for the released radioactivity, using gamma-counter (Beckman, Gamma-4000). The maximum release of radioactivity was determined by incubating the lacrimal gland cells with the same volume of saponin solution (0.25% in water) with 1 mM EDTA, and the spontaneous release was determined by incubating the target cells with medium alone. The specific cytotoxicity was calculated according to the following formula: specific cytotoxicity (%) = cpm (sample) – cpm (spontaneous)/cpm (maximum) – cpm (spontaneous) X 100.

Complement-Dependent Antibody-Mediated Cytotoxicity (CDAC) Test

When the animals were killed, blood was collected by cardiac puncture. The sera obtained were heat-inactivated for 30 min at 56°C and serial twofold diluents were made with the cytotoxicity medium, ranging from 8- to 128-folds. Then, 0.05 ml of the diluent of each serum was mixed with an equal volume of 51Cr-labeled target cell suspension (10^4 cells/well), prepared as described, and incubated for 30 min at 37°C. One-tenth ml of guinea pig complement (1:10 diluted with the cytotoxicity medium) was added to the wells, and the plates were incubated for another 30 min at 37°C. For the controls, target cells were incubated with either eightfold diluted sera
Table 1. Spleen cell-mediated cytotoxicity to lacrimal gland acinar cells

<table>
<thead>
<tr>
<th>Mice examined</th>
<th>Age (months)</th>
<th>Specific cytotoxicity (%)</th>
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<tbody>
<tr>
<td>BALB/c</td>
<td>6</td>
<td>4.4 ± 0.5* (N = 7)</td>
</tr>
<tr>
<td>NZB/W</td>
<td>2</td>
<td>6.1 ± 0.4 (N = 13)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.1 ± 0.5 (N = 9)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5.2 ± 1.0 (N = 9)</td>
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</tbody>
</table>

* The values represent the mean of means.
† Number of mice examined.

or complement alone. Finally, the plate was centrifuged at 100 g for 10 minutes to sediment the target cells and 0.1 ml of the supernatant medium was collected for counting radioactivity. The specific cytotoxicity was calculated in the same way as already described.

Absorption of Pooled Positive Sera by Various Organ and Tissues

The sera of aged NZB/W mice, positive for cytotoxic antibody to lacrimal gland cells, were pooled and used for the absorption study. The absorption of the sera was done according to the method of Harbeck et al. Brain, lacrimal glands, salivary glands, liver, and kidney were obtained from 2-month-old NZB/W mice and were homogenized separately in F-12 medium. The homogenate was washed twice with the cytotoxicity medium, and the pellet was used for the following absorption study. Spleen and thymus cell suspension were prepared by being minced and squeezed in F-12 medium and red blood cell suspension was obtained from heparinized cardiac blood. One hundred microliters of undiluted pooled serum was mixed with an equal volume of pelleted tissues or cells and incubated at 4°C for 1 hour. These absorbed sera were subjected to complement-dependent antibody-mediated cytotoxicity assay at the dilution of 1:4.

Fractionation of Pooled Positive Sera by Gel Filtration

A Sephadex G-150 column (1.6 × 90 cm) was equilibrated with 0.1 M phosphate-buffered saline (PBS, pH 7.2). One milliliter of pooled sera from aged NZB/W mice, which were found to be cytotoxic to lacrimal gland cells, was applied to this column and fractionated at the flow rate of 7 ml/hr. Three peaks were obtained in a total of 15 fractions, each of which consisted of 5 ml. Ouchterlony's double immunodiffusion test was done for all the fractions, using rabbit serum anti-mouse IgG as well as IgM (Cappel Laboratory; Cochranville, PA). IgM was present mainly in fraction 5 and scarcely in fraction 6. On the other hand, IgG was found largely in fractions 6–9 with a trace amount detectable in fraction 5. Consequently, fractions 5–9 were dialyzed against the cytotoxicity medium overnight at 4°C and used for CDAC test at the dilution of 1:4 against the original volume of the sera.

Histopathology of Lacrimal Glands of NZB/W Mice

Both exorbital lacrimal glands were excised when the animals were killed and fixed in the buffered formalin. The specimens were embedded in paraffin, cut in 7 μm thickness, and stained with hematoxylin and eosin (H & E). The section of both lacrimal glands was checked for the number of the infiltrative foci per five fields at ×100 magnification. The cluster consisting of more than 50 lymphocytes was considered as one focus.

Results

Isolation of Lacrimal Gland Cells

Lacrimal gland cells were successfully isolated from exorbital lacrimal glands by a combination of chelation with EDTA, and digestion with collagenase and hyaluronidase. The cytocentrifuged specimen of isolated lacrimal cells consisted predominantly of lacrimal acinar cells. The viability of the isolated cells was between 80–90% and the yield of the cells was 1–2 × 10^5 cells per gland. The isolated lacrimal gland cells could not be maintained for long in tissue culture even in the presence of secretagogue. In fact, the cell viability dropped to around 40% the next day, and the cells became degenerated totally within 3 days. Therefore, we decided to use the lacrimal cells as a target cell immediately after the isolation. In this way, the spontaneous release of the target cell after 4 hr of incubation ranged between 10–15%.

Cytotoxicity Test to Lacrimal Gland Cells

The results of spleen cell-mediated cytotoxicity test against lacrimal gland cells is shown in Table 1. There was no statistically significant difference in the cytotoxicity among the NZB/W mice with the age of 2, 5, and 8 months, and the values ranged between 4–9%. The spleen cells from aged BALB/c mice, an H-2 identical allogeneic control mouse, also were found to have no significant cytotoxicity (Table 1). There was mild splenomegaly in 5- and 8-month-old NZB/W mice.

The presence of serum cytotoxic antibody against lacrimal gland cells was investigated in the same
Table 2. Complement-dependent antibody-mediated cytotoxicity to lacrimal gland acinar cells

<table>
<thead>
<tr>
<th>Mice examined</th>
<th>Age (months)</th>
<th>Specific cytotoxicity (%) to NZB/W lacrimal cells</th>
<th>BALB/c lacrimal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>6</td>
<td>6.6 ± 1.1* (N = 6)</td>
<td>Not done</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.9 ± 1.9 (N = 13)</td>
<td>0.4 ± 1.3 (N = 5)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>18.1 ± 4.7 (N = 9)</td>
<td>6.0 ± 1.4* (N = 9)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>35.8 ± 10.7£ (N = 9)</td>
<td>13.2 ± 3.7£ (N = 9)</td>
</tr>
</tbody>
</table>

* Tie values represent the mean of specific cytotoxicity ± standard error of means.
† The number of mice examined.
‡ Significant from 2-month-old NZB/W mice (P < 0.025); §P < 0.005; \( P < 0.025 \); \( P < 0.01 \), by Student’s t-test.

animals that had been evaluated for spleen cell-mediated cytotoxicity. Serum was taken from both NZB/W and BALB/c mice. As shown in Table 2, cytotoxic effects on the target cells was detectable with the serum of aged NZB/W mice but not with the serum of aged BALB/c mice. As far as NZB/W mice are concerned, the mean level of cytotoxicity against syngeneic targets increased significantly with aging.

The incidence of the number of animals, which showed the cytotoxicity higher than 10%, also was increased. The incidence was two out of thirteen (15%), seven out of nine (78%), and six out of nine (67%), for 2-, 5-, and 8-month-old animals, respectively. There was a statistically significant difference in the level of specific cytotoxicity between 2- and 5-month-old (P < 0.025) as well as 2- and 8-month-old NZB/W mice (P < 0.005). On the other hand, the sera from aged BALB/c did not react with these targets. There was no significant level of the cytotoxicity when the target cells were incubated with either serum or complement alone. The positive sera from NZB/W mice similarly lysed the lacrimal gland cells isolated from BALB/c mice. Again, the cytotoxicity of sera from 5- and 8-month-old NZB/W mice was increased significantly as compared with 2-month-old animals (P < 0.01 and P < 0.025, respectively).

Histopathology of the lacrimal glands of the same animals was checked for infiltration. No infiltration was observed in 2-month-old NZB/W mice; however, eight out of nine (5-month-old animals) and nine out of nine animals (8 months old) showed infiltrative foci.

Characterization of Cytotoxic Autoantibody

To determine the class of these autoantibodies, we fractionated the pooled positive sera from NZB/W mice by gel filtration with Sephadex G-150. Of three peaks obtained, the first one was enriched with IgM (fractions 5, 6), and the second one with IgG (fractions 7-9) as demonstrated by Ouchterloney’s double immunodiffusion test. As Figure 1 shows, the high cytotoxic activity was recovered in the fraction 5, strongly indicating that these antibodies were of IgM class.

The tissue absorption test was performed to check the cross-reactivity of these autoantibodies with other organs. The results showed that the cytotoxic activity was abolished markedly by lacrimal glands and salivary glands, and moderately eliminated by the kidney. Any other organs or cells such as brain, liver, spleen, thymus, or erythrocytes could not affect the cytotoxicity (Fig. 2).

Discussion

New Zealand mice (NZB and NZB/W) are the strains most commonly studied for autoimmune dis-
globulins as well as C3 components were deposited to the site of the reaction with the help of complement. This is not surprising, although we cannot stipulate the specific common antigen did exist in both glands, and that the pathologic significance of these autoantibodies is not known.

We have shown in the present study that anti-lacrimal gland cytotoxic autoantibodies of the IgM class were increased in the sera of aged NZB/W mice. The level and incidence of such antibodies increased significantly with age. Even at the age of 2 months, a considerable level of cytotoxic antibodies was detectable in a few animals. This is not surprising, however, considering the fact that abnormal early maturation of B-cells occurs immediately after birth in these animals.17,18 These antibodies were also capable of lysing the lacrimal gland cells obtained from BALB/c mice, an H-2 identical allogeneic strain, suggesting that tissue-associated antigen might be involved as a target. The result of the tissue absorption study was of much more interest, since the salivary gland, in which a mononuclear cell infiltration occurs in somewhat the same manner as it does in the lacrimal gland, could almost abrogate the cytotoxic activity of the sera. This would indicate that a certain common antigen did exist in both glands, and that this particular autoantibody may be responsible for the salivary gland involvement. Also, there is a possibility that the corresponding antigen might be the product of a virus that is harbored by both glands.19 At present, the role of anti-lacrimal gland autoantibodies in the pathogenesis of cellular infiltration of the lacrimal gland is unclear. One possible assumption is that these antibodies, bound to an antigen in the lacrimal gland cells, could attract inflammatory cells to the site of the reaction with the help of complement. In fact, the finding of deLuise et al10 that immunoglobulins as well as C3 components were deposited in the periductal areas of the lacrimal gland might be of interest, although we cannot stipulate the specific antigens against which such deposited immunoglobulins were directed. Although some reports have suggested the possibility that cell-mediated immunity may play a role in the pathogenesis of the cellular infiltration21-23 of lacrimal and salivary glands, there was no significant level of spleen cell-mediated cytotoxicity to lacrimal gland cells in any animals of any age in our study. Although it may be possible that suppressor cells masked the cytotoxicity in vitro, cell-mediated immunity would not appear to be active in mediating injury. A similar attempt to demonstrate the cellular cytotoxicity to the cultured salivary gland cells in patients with Sjögren's syndrome has failed.24 Thus, anti-lacrimal gland antibodies may be responsible for the tissue injury to the lacrimal gland. However, their presence may simply be the consequence of antibody production in response to damaged lacrimal gland cells. It is possible to obtain monoclonal antibodies to lacrimal gland cells by producing a hybridoma with the spleen cells of aged NZB/W mice, since Eliat et al have succeeded in producing monoclonal antibody to mRNA.20 In subsequent experiments, therefore, passive transfer of antilacrimal gland antibody would be an ideal means of resolving this crucial question.

Key words: Sjögren's syndrome, autoimmune disease, NZB/W mice, autoantibody, cytotoxicity, lacrimal gland

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References


