Experimental Autoimmune Dacryoadenitis

II. Harderian Gland Disease in the Rat

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Experimental autoimmune dacryoadenitis was induced in 100% of Lewis rats by immunization with a KCl extract of Harderian gland in complete Freund's adjuvant (CFA), providing that the animals had received simultaneously i.v. injection of killed Bordetella pertussis. No significant pathological changes in the Harderian gland were observed in control animals immunized with KCl extracts of lacrimal or salivary glands. Gel filtration of the KCl extract on Sephacryl S-300 column yielded three protein fractions. Fraction II (MW = 50–100K) induced severe Harderian gland disease following a single injection of 2.0 mg protein in CFA plus pertussis. The initial lesions consisted of multiple focal infiltrates of mononuclear cells. Later, the inflammatory process assumed a more granulomatous form, with significant contribution by epithelioid and giant cells. In contrast, Lewis rats immunized with Harderian gland fractions I or III proteins, or with extracts of lacrimal or salivary gland, showed little or no inflammatory lesions. These data suggest that Harderian gland contains unique tissue-specific autoantigen(s) capable of inducing autoimmune granulomatous dacryoadenitis in the rat. Invest Ophthalmol Vis Sci 28:276–280, 1987

The rat has three major tear glands: the Harderian gland, an exorbital lacrimal gland, and an intraorbital lacrimal gland. Harderian and lacrimal glands have a common embryological origin and similar blood vascular system,1,2 but differ markedly in their physiological and immunological functions. The Harderian gland is a large acinotubular gland located medioposteriorly in the orbit, whose primary function is to lubricate the nictitating membrane with a sebaceous and mucoid material.3,4 One of the characteristic features of this gland is the presence of porphyrins, and it may serve as an extraretinal receptor of light, since it appears to exert a regulatory diurnal effect on pineal gland function in rats and hamsters.5,6 By contrast, the lacrimal gland is a compound tubuloalveolar secretory gland whose aqueous secretion contributes the greatest amount to the tear volume, consisting mainly of water, electrolytes, and proteins.7 The lacrimal gland has recently been shown to contain larger numbers of IgA-producing cells in the interstitium than does the Harderian gland, which suggests that the former may be the primary site of the secretory immune system of the eye.8

We have shown that an organ-specific autoantigen(s) exists in the lacrimal gland of the rat, capable of inducing spontaneous autoimmune lymphoproliferative lacrimal dacryoadenitis, while sparing the Harderian gland.9 The studies described herein demonstrate that rat Harderian gland also contains a unique, tissue-specific autoantigen(s) able to induce an autoimmune dacryoadenitis limited to the Harderian gland and different in its histopathology from that of the lacrimal gland disease.

Materials and Methods

Animals

Female Lewis rats weighing 150–175 g were obtained from Charles River Laboratories, Wilmington, MA, and were used in all experiments.

Preparation of Harderian Gland Antigen

The procedures employed were similar to those described previously for lacrimal gland antigens.9 Rat Harderian glands were excised from fresh eyes and stored at −20°C until use. A 20% suspension of the glands was prepared in 0.01 M Tris-HCl buffer at pH 7.8, homogenized, and centrifuged at 40,000 g for 30 min. The supernatant (termed “saline extract”) was decanted, and sterilized through Millex-HA (0.45 μm, Millipore, Bedford, MA). The pellet remaining after

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centrifugation was suspended in Tris-HCl buffer containing 3M KCl (2 ml of solvent per gland), and extracted as previously described (termed "crude KCl extract"). In addition, KCl extracts of exorbital lacrimal gland or submaxillary gland were prepared in a similar manner for use as control antigens. The KCl extract of Harderian gland was applied to a calibrated 2.5 X 95 cm Sephacryl S-300 column equilibrated in phosphate-buffered saline (PBS), and eluted at a flow rate of 60 ml/hr at 4°C. Fractions were collected in 10-ml volumes and pooled in three fractions according to the 280 nm profile (Fig. 1). Crude extracts were concentrated to 10 mg/ml, and chromatographic column fractions to 2 mg/ml on an Amicon YM-10 membrane (Amicon Corp.; Danvers, MA). All protein determinations in this study were performed by using the dye-binding Bio-Rad protein microassay (Bio-Rad Lab.; Richmond, CA).

Sensitization of Animals

Different groups of Lewis rats were sensitized with a single dose of either 10 mg of the saline extract of Harderian gland, 10 mg of the crude KCl extract, or 2.0 mg of each of the three fractions obtained from the chromatographic column. The antigens were emulsified (1:1) in complete Freund's adjuvant (CFA) supplemented with 2 mg/ml *Mycobacterium tuberculosis* H37Ra strain (Difco, Detroit, MI). All four footpads were injected, the total inoculum volume being 0.4 ml. In some experiments, the rats were also injected i.v. with 5 X 10^10 Bordetella pertussis (BP) at the same time that antigen was inoculated. Killed pertussis vaccine (lots 55-91B) was obtained from the Michigan Department of Health, Lansing, MI.

Assessment of Inflammatory Disease

Groups of animals were killed at intervals from 10–60 days after sensitization. The Harderian, exorbital lacrimal, and salivary glands were removed and processed for histologic examination, and the severity of dacryoadenitis was graded histologically on a scale of 0–3+, as described previously.9

These investigations conform to the ARVO Resolution on the Use of Animals in Research.

Results

Induction of Autoimmune Dacryoadenitis With Crude Extracts

The incidence and specificity of autoimmune dacryoadenitis produced in groups of five to ten Lewis rats after a single immunization with Harderian gland or control antigens were assessed on day 25 and are summarized in Table 1. Injection of rats with saline extract

Table 1. Induction of autoimmune Harderian dacryoadenitis in the rat using crude or partially purified organ extracts

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen</th>
<th>Mode of immunization*</th>
<th>Incidence†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>CFA + pertussis</td>
<td>0/5</td>
</tr>
<tr>
<td>2</td>
<td>Harderian saline extract</td>
<td>CFA</td>
<td>0/5</td>
</tr>
<tr>
<td>3</td>
<td>Harderian saline extract</td>
<td>CFA + pertussis</td>
<td>0/5</td>
</tr>
<tr>
<td>4</td>
<td>Harderian KCl extract</td>
<td>CFA</td>
<td>3/10</td>
</tr>
<tr>
<td>5</td>
<td>Harderian KCl extract</td>
<td>CFA + pertussis</td>
<td>10/10</td>
</tr>
<tr>
<td>6</td>
<td>Lacrimal KCl extract</td>
<td>CFA + pertussis</td>
<td>0/5</td>
</tr>
<tr>
<td>7</td>
<td>Submaxillary KCl extract</td>
<td>CFA + pertussis</td>
<td>0/5</td>
</tr>
<tr>
<td>8</td>
<td>Sephacryl fraction I</td>
<td>CFA + pertussis</td>
<td>0/16</td>
</tr>
<tr>
<td>9</td>
<td>Sephacryl fraction II</td>
<td>CFA + pertussis</td>
<td>16/16</td>
</tr>
<tr>
<td>10</td>
<td>Sephacryl fraction III</td>
<td>CFA + pertussis</td>
<td>0/16</td>
</tr>
</tbody>
</table>

* The antigen (10 mg of the crude KCl extract or 2 mg of the Sephacryl fraction) was emulsified in adjuvant and injected in the footpads, whereas pertussis was administered i.v.
† Numbers indicate the number of animals positive/total number tested.

Histologic assessment of dacryoadenitis was made 20–40 days after sensitization and was recorded as positive when multiple foci occupied more than 10% of the gland.
Table 2. Dacryoadenitis induced in rats after sensitization with chromatographic fraction II of the Harderian gland

<table>
<thead>
<tr>
<th>Days after immunization</th>
<th>Harderian* gland</th>
<th>Exorbital lacrimal gland</th>
<th>Salivary gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
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<td>0/4</td>
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<tr>
<td>15</td>
<td>3/4</td>
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</tr>
<tr>
<td>60</td>
<td>3/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* Numbers indicate number of animals positive/total number tested. A lesion score of grade 2+ or greater was judged to be positive.

of Harderian gland in CFA alone (group 2) or with pertussis (group 3) yielded no significant inflammatory lesions above control levels. In those rats receiving the crude 3M KCl extract of the homogenate sediments in CFA alone (group 4), three of ten showed significant pathologic changes in their Harderian glands. However, all ten rats immunized with the same extract in CFA, together with the additional i.v. injection of pertussis (group 5), developed severe Harderian gland disease. In contrast, control rats immunized with 3M KCl extracts of exorbital lacrimal gland (group 6), or submaxillary gland (group 7) in CFA plus pertussis, did not develop significant pathologic changes in their Harderian glands. They showed only mild, nonprogressive inflammatory foci similar to those seen in rats receiving only CFA and pertussis (group 1), presumably due to the nonspecific effects of the vehicle employed.

Induction of Dacryoadenitis With Partially Purified Fractions

The 3M KCl extract of the Harderian gland was separated into three protein fractions by gel filtration on a Sephacryl S-300 column (Fig. 1). Peak I contained the highest molecular weight (MW) proteins found nearest the void volume. Fraction II contained proteins of approximately 50,000-100,000 MW. Peak III contained proteins smaller than about 45,000 MW. The proteins in each of the three fractions were used to immunize separate groups of 16 Lewis rats, in an attempt to localize the active autoantigen(s).

Separate groups of rats were sensitized with 2 mg of the Sephacryl fractions in CFA with simultaneous i.v. injection of pertussis. They were assessed histologically for signs of Harderian dacryoadenitis between 20 and 40 days after sensitization. As Table 1 makes clear, only fraction II produced disease. The occasional animal sensitized with fraction III showed small focal nongranulomatous lesions, primarily lymphocytic, involving 5% of the gland or less, perhaps due to overlapping contamination by the active peak II material.

Specificity of Harderian Dacryoadenitis

A large group of rats was sensitized with 2 mg of the active peak II antigen in CFA with i.v. pertussis, and their Harderian, exorbital, and salivary glands were examined at intervals from 10-60 days thereafter. As can be seen in Table 2, most animals developed Harderian dacryoadenitis from day 15 onward, but in no instance was a concomitant salivary or lacrimal gland inflammation noted.

Characteristics of Harderian Dacryoadenitis

The 32 Lewis rats immunized with a single dose of 2.0 mg of fraction II proteins in CFA plus pertussis, as shown in Table 2, were examined clinically and histologically for changes in their glands at intervals from 10-60 days after immunization. The Harderian gland appeared to be grossly normal during the first 15 days after immunization. Over the following weeks, the Harderian glands of all immunized animals became enlarged and swollen, and showed small focal areas of hemorrhage. By the end of week 7, they were reduced in size and presented a firm consistency. The exorbital lacrimal glands and salivary glands of these rats appeared grossly normal.

No lesions were observed histologically at 10 days after sensitization, whereas mild lesions were found in 3 of 4 rats killed on day 15. The lesions at this time consisted of small, multifocal interstitial infiltrates that appeared to surround certain acini (Fig. 2). The in-
Inflammatory infiltrates consisted of lymphocytes and macrophages. In some cases lymphocytes predominated, but in others macrophages were more numerous. There was little or no destruction of glandular tissues, and the bulk of the gland appeared normal.

The interstitial lesions rapidly became more extensive, and by 20–30 days after immunization there was a marked change in the character of the lesions. They were now more granulomatous in nature, with numerous epithelioid cells surrounded by a mantle of lymphocytes. There was appreciable variability in the severity of disease, although by this time all animals had developed dacryoadenitis. Some had milder 2+ lesions, whereas others showed more extensive and active 3+ lesions. The number of epithelioid cells seemed to be related to the severity of disease, increasing in proportion to the degree of inflammation and of tissue destruction. In the milder 2+ lesions, multiple compact bands of fibrous tissue containing lymphocytes and macrophages were interspersed through the glandular tissue, with some epithelioid cells in evidence. In the more severe cases, there was considerable fibrosis and extensive inflammatory cell infiltration, with destruction of glandular architecture (Figs. 3, 4). Numerous epithelioid cells and occasional giant cells were seen, surrounded by lymphocyte and/or mononuclear collections. Phagocytosis of cellular debris, focal necrosis, and occasional hemorrhage into acini were also noted in some areas.

The active granulomatous reaction was no longer observed 50–60 days after immunization. There was a marked decrease in the overall extent of disease. Many of these mild lesions consisted of multiple small focal collections of chronic inflammatory cells, with macrophages somewhat more frequent than had been observed earlier, although lymphocytes were still present. The characteristic feature at this stage was resolution of the earlier destructive process, with pigmented material lying over the glandular tissue. The latter may be due to acinar destruction, with the release of their reddish-brown porphyrin pigment.

The exorbital lacrimal glands of those rats with severe Harderian gland disease showed only minimal inflammation, hardly above that seen in control animals. These lesions were characterized by diffuse and irregularly distributed interstitial mononuclear cell infiltrates, affecting less than 5% of the gland. No histologic abnormalities could be found in the salivary glands of those rats with severe Harderian gland dacryoadenitis.

**Discussion**

There have been contradictory reports on attempts to produce Harderian gland dacryoadenitis experimentally. Pisarev et al. produced severe lesions in guinea pigs injected with crude homogenates of Harderian gland in CFA, whereas Singh and McKenzie, employing a similar approach, were unable to produce this effect in mice. We were also unable to induce Harderian gland lesions by sensitizing Lewis rats with the supernatants of simple gland homogenates in CFA alone, or with *Bordetella pertussis*, a bacterium known to exert a strong adjuvant effect on a number of different model systems for autoimmune disease. We have shown in the present studies, however, that further extraction with 3M KCl of the insoluble sediment from...
those crude homogenates recovers a specific antigen(s) capable of inducing severe Harderian gland disease in the Lewis rat. KCl extraction is a standard procedure employed to release proteins from cell membranes and other insoluble aggregates. Autoimmune Harderian dacryoadenitis can be induced consistently with this preparation, but only when it is incorporated in CFA and accompanied by simultaneous i.v. injection of pertussis (Table 1).

The Harderian gland disease that we have produced in this study appears to be organ-specific. Neither the lacrimal nor salivary glands of animals sensitized with Harderian antigens showed pathologic changes. Further, sensitization of control rats with analogous extracts of either lacrimal or submaxillary glands caused no Harderian gland pathology.

Preliminary efforts to purify the active Harderian gland antigen(s) by gel filtration of the KCl extract showed it to localize in a fraction containing proteins with molecular weights in the range of 50-100,000 daltons. This antigen(s) appeared to differ from the one(s) responsible for lacrimal gland dacryoadenitis, which was found in the chromatographic fraction containing proteins with MW = 10-55K.

The histopathology of the Harderian gland lesions also differed markedly from that of the lacrimal gland dacryoadenitis. The initial lesions in the Harderian gland, seen at about 15 days after immunization, were composed of multiple focal interstitial mononuclear cell infiltration, with the bulk of the gland appearing normal. But as the inflammatory process increased in severity and extent (reaching a maximum in 20-30 days), a significant epithelioid cell component was seen. The more severe the degree of dacryoadenitis, the more marked was the granulomatous nature of the lesion. In contrast, the lacrimal gland study showed that the inflammatory lesions in the lacrimal gland were predominantly lymphoid and nongranulomatous, even in the most severely involved cases.

It is unclear why granulomatous inflammation should develop in autoimmune Harderian gland disease and not in autoimmune lacrimal gland disease. In some instances, such as S-antigen-induced uveoretinitis, it is the dose of immunizing antigen that appears to determine the cell types involved in the inflammatory response, presumably reflecting differing levels of host sensitization and thus intensity of response. However, even the most severe types of autoimmune thyroid disease rarely become granulomatous and may progress to fibrosis and scarring without passing through an epithelioid cell phase. In contrast, even mild inflammatory responses to such organisms as the tubercle bacillus are characterized by granuloma formation, a feature generally ascribed to the lipid components of the stimulus. A similar biochemical difference may be responsible for the varying nature of lacrimal and Harderian gland immunopathology, since the former produces a predominantly aqueous secretion whereas the latter secretes a more sebaceous product.

These studies demonstrate that a reproducible autoimmune dacryoadenitis may be produced specifically either in the lacrimal or Harderian glands of the rat. The establishment of these experimental models will now permit the isolation and identification of the autoantigens involved and their localization within the gland structures. Further studies of these experimental models, including the use of other inbred strains and of passive transfer experiments, may contribute to our understanding of the pathogenesis of analogous autoimmune processes in man.

Key words: dacryoadenitis, Harderian gland autoimmunity, lacrimal gland autoimmunity, autoimmune dacryoadenitis

References