Experimental Autoimmune Dacryoadenitis: Purification and Characterization of a Lacrimal Gland Antigen

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A soluble lacrimal gland antigen (LG-Ag) capable of inducing experimental autoimmune dacryoadenitis (EAD) in SJL/J mice has been purified from bovine lacrimal gland extracts by a combination of ion exchange and gel-filtration chromatography. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a single band with a mobility corresponding to a molecular weight of 45,000 daltons. Antibodies raised in guinea pigs against purified LG-Ag were used to confirm the tissue-specificity and to assess the cellular localization of LG-Ag within tissues. Anti-LG-Ag serum reacted with the crude lacrimal gland extracts but not with the extracts of bovine submaxillary gland, parotid gland, or retina.

As shown by immunofluorescent staining, LG-Ag appeared to be localized on the murine lacrimal ductal epithelial cells but was lacking in the salivary ducts. LG-Ag induced EAD that was characterized by infiltration of mononuclear cells around the ducts and associated vasculature, accompanied by extensive damage of the acinar cells. The lesions induced were tissue-specific, in that SJL/J mice immunized with LG-Ag had intense lymphocytic infiltrates in lacrimal gland, but had no significant lesions in their salivary and hardarian glands. The availability of a purified LG antigen can facilitate the further analysis of pathogenetic mechanisms in EAD. Invest Ophthalmol Vis Sci 33:2029–2036, 1992

Severe chronic inflammatory reactions in the lacrimal gland (LG) lead to decreased tear flow and dry eye, which are important clinical problems in primary Sjögren’s syndrome. However, the pathogenesis of these lesions is unclear. The development of experimental animal models may help to elucidate the pathogenesis of some of these human disease processes thought to be of autoimmune origin. In earlier studies, we successfully produced severe dacryoadenitis in Lewis rats by immunization with homologous LG extracts.1,2 Those studies suggested that the LG contains its own unique tissue-specific autoantigen(s) capable of inducing experimental autoimmune dacryoadenitis (EAD) in the rat. Those studies also prompted us to isolate and purify the autoantigen(s) responsible for the induction of EAD.

The use of a purified antigen is important if the basic pathogenetic mechanism of a tissue-specific autoimmune disease is to be understood fully. The S-antigen is a good example of how the use of a purified antigen in producing experimental autoimmune uveoretinitis (EAU) can advance our knowledge of immunopathologic mechanisms and can mediate our approach to the understanding and therapeutic consideration of some forms of human uveitis.3 The present study is the first in a series that deal with antigens isolated from the LG. In this study, we report the purification, characterization, and immune reactivity of a tissue-specific LG antigen (designated LG-Ag). Immunization of SJL/J mice with the purified LG-Ag induced severe dacryoadenitis with lymphocytic infiltration in their LG.

Materials and Methods

Animals

Female SJL/J mice 8–10 wk old were obtained from Jackson Laboratories (Bar Harbor, ME) and were used as immunization host in all experiments. These investigations conformed to the ARVO Resolution on the Use of Animals in Research.

Assay for EAD-Inducing Activity

Female SJL mice were immunized with the various protein fractions in complete Freund’s adjuvant (CFA). To prepare the inoculum, various amounts of each protein fraction at different stages of purification were emulsified (1:1) in CFA supplemented with 2 mg/ml Mycobacterium tuberculosis H37 Ra strain (Difco, Detroit, MI). The inoculum (0.2 ml) was subcutaneously distributed in the flanks at four sites. Animals were killed 30 d after immunization. The LG,
harderian glands (HG), and submaxillary glands (SG) were removed and processed for histologic examination. The severity of dacryoadenitis was graded histologically on a scale of 0 to 4+, as previously described.\textsuperscript{1,2} In grade 1+, up to 5% of the LG sections were infiltrated with mononuclear cells; in grade 2+, between 5 and 15%; in grade 3+, between 15 and 35%; and in grade 4+, between 35 and 50% of the LG sections were infiltrated.

Purification of LG-Ag

\textbf{Step 1—Preparation of LG extracts:} The procedures employed were similar to those described previously.\textsuperscript{1,2} Bovine LG were obtained from a local slaughterhouse and stored at \(-20^\circ\text{C}\) until used. The glands were subsequently fragmented into small pieces and suspended in a 10% (weight/volume) suspension in phosphate buffered saline (PBS). The suspension was stirred slowly for 24 hr at \(4^\circ\text{C}\) and centrifuged. The supernatant was decanted and saved. The pellet was extracted with 3 mol/l KCl in PBS for 24 hr at \(4^\circ\text{C}\) and centrifuged. The supernatant was dialyzed in the cold against several changes of PBS. The two supernatants then were pooled and concentrated to 20 mg of protein per ml on an Amicon YM-10 membrane (Amicon Corp., Danvers, MA). All protein determinations in this study were performed with the dye-binding protein assay (Bio-Rad, Richmond, CA) with bovine serum albumin as standard.

\textbf{Step 2—Anion exchange chromatography:} The LG extracts were dialyzed against several changes of 0.01 mol/l phosphate buffer, pH 8.0 (DE buffer). Sixty milliliters of the extracts containing 1.2 gm proteins were applied to a 4 \(\times\) 20 cm column of DE-52 (Whatman, Clifton, NJ) equilibrated with DE buffer. The column was run at room temperature at a flow rate of 20 ml/hr.

\textbf{Step 3—Gel filtration chromatography:} The active DEAE fraction was concentrated down to 5.0 ml and applied to a 2.5 \(\times\) 120 cm Sephadex G-75 superfine column (Pharmacia, Piscataway, NJ) previously equilibrated with PBS. The column was calibrated with various known molecular weight markers (Sigma, St. Louis, MO) in PBS at a flow rate of 20 ml/hr at \(4^\circ\text{C}\). The protein peaks were pooled according to the 280 nm profile.

\textbf{Step 4—Hydroxylapatite Chromatography:} The active fractions from the Sephadex G-75 column were pooled, concentrated, and dialyzed against several changes of 0.02 mol/l potassium phosphate buffer, pH 6.8 (HA buffer). The sample was applied to a 1.5 \(\times\) 30 cm column of Bio-gel HT (Bio-Rad) equilibrated with the HA buffer. After washing with the starting HA buffer, a linear gradient consisting of 150 ml 0.02 mol/l and 150 ml 0.5 mol/l potassium phosphate buffer (pH 6.8) was applied to the column. The material was eluted at \(4^\circ\text{C}\) at a flow rate of 20 ml/hr. Four-milliliter fractions were collected and monitored for absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli\textsuperscript{4} using slab gel of 7.5% polyacrylamide. Samples of 5–50 \(\mu\)g protein were dissolved in 1% SDS in 0.05 mol/l TRIS buffer (pH 6.8), 10% glycerol, and 5% dithiothreitol. Samples were treated for 5 min with boiling water. Protein standards of known molecular weight (MW-SDS-200; Sigma, St. Louis, MO) were treated in the same fashion. Electrophoresis was carried out at room temperature at 25 mA/gel until the bromophenol blue marker reached the end of the gel. The gels then were stained with coomassie brilliant blue, destained, and stored in 15% methanol and 10% acetic acid.

Indirect Immunofluorescence

Hartley guinea pigs were intradermally injected at multiple sites of the back with 2.5 mg of purified LG-Ag in CFA. Four weeks after sensitization, the animals were challenged with an equivalent dose of the antigen in incomplete Freund’s adjuvant. Antisera were collected 2 wk after booster.

Indirect immunofluorescent technique was used to locate the antigen in sections of tear glands. The LG, HG, or SG from SJL mice were sectioned at 4 \(\mu\)m in a cryostat. The tissue sections were fixed in acetone for 5 min and incubated for 1 hr at room temperature with normal rabbit serum (1/10 dilution in PBS) to block nonspecific absorption of the tissue. The slides were subsequently incubated with antiserum against LG-Ag and then with a fluorescein-labeled rabbit anti-GP IgG (Sigma) after the standard washing procedures with PBS.

Immunodiffusion

Ouchterlony analysis was used to assess the organ specificity of LG-Ag. The diffusion plates were carried out in 1% agarose made up in 0.05 mol/l borate buffered saline (pH 8.2), using 25 \(\mu\)l wells 10 mm apart. Bovine submaxillary and parotid glands and retina were extracted with PBS and 3 mol/l KCl, as described above, dialyzed, and concentrated (10 mg/ml) for use as control antigens. Precipitation lines, which
developed after 48 hr incubation in a moist chamber at room temperature, were stained with amide black and photographed.

Results

Purification of LG-Ag

**DEAE ion exchange chromatography:** The crude LG extracts were subjected to ion change chromatography on a DE-52 column. The column was eluted with stepwise increasing concentrations of NaCl and separated into five fractions (Fig. 1). The proteins in each of the five fractions were used to immunize separate groups of SJL mice in an attempt to localize the active antigen(s). Figure 1 summarizes the data on the incidence of EAD obtained in SJL mice after a single sensitizing dose of 0.2 mg of each of the five fractions emulsified in CFA. Immunization of mice with fraction I (eluted with 0.05 mol/l NaCl) caused three of five to show significant pathologic changes in their LG. However, the vast majority of SJL mice (four of five) immunized with fraction IV (eluted with 0.2 mol/l NaCl) in CFA developed severe dacryoadenitis. The lesions were characterized by multifocal mononuclear cell infiltrates and acinar cell damage. In contrast, the LG of those mice that received other DEAE fractions contained only occasional minute foci of chronic inflammatory cells. There were usually one or two of these foci and never more than three. They appeared to occupy less than 5% of the gland (grade 1+ dacryoadenitis or less). Therefore, we decided to purify fraction IV further because of its immunopathogenic activity. The yield of protein in the fraction IV was approximately 25–30% of the crude extracts applied to the DEAE column.

**Sephadex G-75 gel filtration:** The active DEAE fraction IV was subjected to gel filtration on a Sephadex G-75 column and separated into three protein peaks (Fig. 2). Only peak III induced severe dacryoadenitis after a single immunization of 0.2 mg protein in CFA, whereas use of column peaks that contained higher molecular weight material failed to produce LG disease. The molecular weight of the eluants was estimated after calibration of the column with known marker proteins. It can be seen in Figure 2 that peak III contained proteins of approximately 45,000 daltons. Approximately 20% of the protein applied to the Sephadex column was recovered in peak III.

**Ion exchange chromatography on hydroxyapatite:** The active peak from the gel filtration step was further purified by hydroxyapatite chromatography. In SJL mice that received injections of 0.1 mg of fraction III
Fig. 3. Hydroxylapatite chromatography of the active G-75 peak III. The column was eluted with 0.02 M potassium phosphate buffer (pH 6.8), and then by a linear gradient of 0.02 M and 0.5 M phosphate buffer (pH 6.8). Immunization of SJL mice with 0.1 mg of fraction III produced severe LG dacryoadenitis.

in CFA, severe LG lesions were found in all (5/5) mice killed on day 30. Only rarely did an animal sensitized with fraction II proteins show mild histologic changes, in contrast to the extensive dacryoadenitis induced by immunization with fraction III protein (hereafter referred to as LG-Ag). It is possible that the mild change was a result of minor contamination of the active LG-Ag material. Immunization of mice with fraction I protein did not induce significant pathologic changes in their LG.

Because of a partial overlap of the neighboring inactive materials, rechromatography of fraction III under identical conditions on a new hydroxylapatite column was necessary. This step led to a symmetrical peak that coincided with the LG-Ag immunopathogenic activity (data not shown) and to greatly increased purity of the protein. However, considerable losses were encountered during this step of chromatography, probably because of the strong binding of LG-Ag to the hydroxylapatite. The protein content of bovine LG crude extracts was approximately 1.2 gm. The LG-Ag-rich fraction obtained after the four-step chromatography yielded approximately 20-30 mg, the equivalent of 2% of the starting materials. This represents a 50-fold purification when related to the initial protein content of the crude extracts.

Molecular Weight Determination

SDS-PAGE was used as an additional criterion of purity as well as an alternative method to gel filtration for molecular weight analysis. The LG-Ag sample was deliberately overloaded, revealing only a single band corresponding to a molecular weight of 45,000 daltons (Fig. 4). This value agreed with the molecular weight range obtained from the gel-filtration step (Fig.

![Molecular Weight Determination](https://example.com/image)

Table 1. Immunopathogenicity of various doses of purified LG-Ag protein

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<th>Dose (µg)</th>
<th>Incidence</th>
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<td>200.0</td>
<td>5/5</td>
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<tr>
<td>100.0</td>
<td>10/10</td>
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<td>50.0</td>
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<td>25.0</td>
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* Amount of LG-Ag protein was emulsified in CFA. SJL mice were immunized with a single dose of LG-Ag as indicated, and sacrificed 30 days after immunization.
† The numbers indicate animals positive/animals tested. A lesion was judged to be positive when multiple foci occupied more than 10% of the LG (grade 2+ dacryoadenitis or greater).
induced EAD in three of five animals. At the lower dose of 25 μg, some mild infiltration of cells was observed, but only one animal exhibited significant changes in the LG. Therefore, it is clear that the minimum dose of LG-Ag capable of inducing severe EAD in all immunized SJL mice is 100 μg protein.

The types of lesions induced in SJL mice by bovine LG-Ag were identical to those produced in Lewis rats by homologous LG extracts.\(^1\)\(^2\) The affected glands most commonly showed an extensive inflammatory cellular infiltration associated with destruction of glandular tissues (Fig. 5A). Nearly all of the inflammatory cells were mononuclear cells, the great majority of which were lymphocytes. While some macrophages also were present, there was no evidence of granuloma formation. In severe cases, acini were replaced by foci of mononuclear cells centered on ducts and some blood vessels (Fig. 5B). The submaxillary, sublingual, and parotid glands of those mice with severe LG dacryoadenitis showed only minimal involve-
ment. These were single, small focal collections of histocytes, and were similar to the mild changes seen in control animals injected with CFA without glandular extracts. Furthermore, the hardarian glands of these animals showed no inflammatory changes above control levels.

**Immunofluorescent Localization of LG-Ag**

An indirect immunofluorescence technique was used to assess the cellular localization of LG-Ag within tissues, as well as to confirm the organ specificity. The anti-LG-Ag antibody gave strong specific fluorescence of lacrimal ductal epithelial cells (Fig. 6a). There was no acinar staining. The antiserum was organ specific, with no fluorescence seen when submaxillary glandular tissues from the same mouse was stained with anti-LG-Ag antibody (Fig. 6b). The preimmune serum did not produce staining of the LG cells.

**Immunodiffusion Analysis**

Antiserum directed against purified LG-Ag was used in immunodiffusion analysis of soluble extracts of bovine tissues. Antiserum to LG-Ag showed only a

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Fig. 6. Indirect immunofluorescent staining of normal SJL mouse LG and SG with guinea pig anti-bovine LG-Ag serum. (A) Fluorescent staining was specific for the lacrimal ductal epithelial cells with no acinar staining. (B) Negative immunofluorescent staining of salivary ducts with the same serum used in (a), which contained lacrimal duct antibody.
single immunoprecipitant band when reacted with LG-Ag and the crude LG extracts. The precipitin line that formed between LG-Ag and antiserum and between the crude extracts and antiserum joined with each other, clearly indicating a high degree of immunologic purity of LG-Ag. In the Ouchterlony test, even with large concentrations of the extracts (1-10 mg/ml) used as antigen and a long (3-4 d) period of observation, there was no positive reaction with the extracts obtained from SG, parotid gland, and retina. The data provided further evidence for the organ-specific nature of LG-Ag. A typical example of an immunodiffusion test is shown in Fig. 7.

Discussion

The objective of the present study was to isolate and purify an LG antigen capable of specifically inducing autoimmune dacryoadenitis. The LG-Ag has been sequentially purified from the crude extracts by DEAE-cellulose chromatography, gel-filtration on G-75, and hydroxylapatite chromatography. It was purified approximately 50-fold, based on the initial protein content of the crude LG extracts. This protein antigen appears to be a single polypeptide with a high degree of homogeneity as determined by reduced SDS-PAGE. It has an apparent molecular weight of 45,000 daltons. The LG-Ag is an effective antigen. As little as 100 μg was capable of inducing severe EAD in all of the SJL mice tested. The dacryoadenitis induced was organ specific: SJL mice immunized with the purified LG-Ag had intense lymphocytic infiltration in their LG, but no significant lesions in their HG and SG. Two immunochemical techniques were employed to test whether LG-Ag was truly organ-specific for LG. Findings confirmed that (1) a single precipitin band was present in immunodiffusion when anti-LG-Ag serum was reacted with the crude LG extracts but not with the extracts of SG, parotid gland, or retina; and (2) LG-Ag, as shown by indirect immunofluorescence, was confined to a single ductal epithelial cell type in the LG. The SG did not contain these LG-Ag determinants.

We previously have shown that EAD can be induced in Lewis rats by immunization with homologous LG extracts in CFA, providing that the animals had received simultaneously intravenous injection of Bordetella pertussis vaccine. Treatment with pertussin is crucial to the successful production of lacrimal lesions in the rat model system. In other words, Lewis rats need pertussin to be converted to good responders that develop severe EAD. Although the differences in susceptibility between strains can be overcome by the use of pertussin, the role of pertussin has not yet been defined. It is perhaps related to histamine factors and increases the permeability of the blood-retina barrier in EAU. In the present model system, EAD was readily induced in mice without pertussin by a single injection of a purified LG-Ag emulsified in CFA. The precise mechanism by which a tissue-specific LG-Ag may break natural tolerance and induce autoimmune dacryoadenitis when injected with adjuvant is not well understood. It may be the result of adjuvant-induced polyclonal activation of the immune system. It is likely that CFA alters the equilibrium between helper and suppressor T cells, resulting in an enhanced CD4+ helper T cell function. It is now clear that specifically autoreactive helper and suppressor cells persist in the healthy body and that the degree of autoreactivity is held in check by a variety of controlling mechanisms. The autoimmunity to LG-Ag leading to the induction of dacryoadenitis is assumed to be a consequence of a distortion in the equilibrium that favors helper activity.

The severity of EAD is governed by the genetic constitution of the animals (Liu et al, manuscript in preparation). Whereas SJL mice with the H-2d haplotype are good responders that develop extensive cellular infiltration of the LG, those with the H-2b or H-2q haplotype are considered poor responders because of little infiltration of the LG. The mouse offers numerous advantages for further in-depth studies of the humoral or cellular events involved in the pathogenesis of EAD, namely because of the availability of monoclonal antibodies to identify T cell subsets and many inbred strains to manipulate the genetic system. Further studies of the present model system, including...
the use of passive transfer experiments, may contribute to our understanding of the pathogenesis of analogous autoimmune processes in humans.

Observations from clinical and laboratory studies suggest the involvement of LG and SG ducts in the autoimmune processes leading to glandular damage in Sjögren’s syndrome.\(^9-11\) This conclusion is supported by the findings of the earliest glandular lesions occurring in the small intralobular ducts and the presence of an autoantibody to the lacrimal and salivary duct cells in some patients with Sjögren’s syndrome. Our experimental model of autoimmune disease may have implications for those forms of dacryoadenitis suspected of having an autoimmune component and associated with lacrimal gland manifestations, namely Sjögren’s syndrome and idiopathic dacryo-adenitis. The histologic and immunologic findings in the mouse model are similar in many respects to that of Sjögren’s syndrome. A chief histologic feature is chronic inflammation surrounding the lacrimal ducts with accumulations of mononuclear cells, accompanied by acinar atrophy. Basis on the immunofluorescent results, LG-Ag is localized in the lacrimal duct cells. Presumably, the LG-Ag protein is exposed in an external position in the target lacrimal ductal epithelial cells, where it is accessible for reaction with autoantibody or sensitized lymphocytes that may mediate the pathologic events. Therefore, LG-Ag may be used clinically as an immunochemical probe to assess the state of presensitization through its reactivity with patient lymphocytes and sera.

**Key words:** dacryoadenitis, lacrimal gland antigen, lacrimal gland autoimmunity, autoimmune dacryoadenitis

**References**