Experimental Induction of Sialadenitis in Rats by Heterologous Anti-Salivary Gland Antibodies

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The purpose of the present study was to investigate the possibility of experimental induction of sialadenitis in rats by heterologous anti-salivary gland antibodies.

Two fractions (Fractions I and II) were separated from rat submaxillary gland by Sephadex-G 200 gel-filtration, and then rabbits were immunized with each fraction.

Although cross-reactivity of anti-FrI antisera obtained from the rabbits against rat liver and rat serum was shown, specific reactivity of both anti-FrI and anti-FrII antisera against rat submaxillary gland was demonstrated by immunodiffusion or the indirect immunofluorescence method. Activity of antibody-dependent cell-mediated cytotoxicity against fresh rat submaxillary gland was also demonstrated using the anti-FrII antisera in vitro.

Sialadenitis with periductal cell infiltrations was induced in the submaxillary glands of many rats injected intraperitoneally with each heterologous antisera. Similar lesions, however, were also induced in the parotid glands, livers, or kidneys of these rats. By a direct immunofluorescence method, granular deposits of rabbit gammaglobulin were detected in cytoplasm of the infiltrating cells and the small vessels around the lesions of the submaxillary glands.

It was suggested, therefore, that the lesions are induced by circulating immune complexes probably formed by non-organ-specific antibodies contaminating each antisera. Salivary gland lesions induced by a similar mechanism were also suggested in patients with Sjögren's syndrome, although the pathological significance in vivo of the anti-salivary gland antibody as an organ-specific antibody could not be confirmed by the present study.

(Key Words: Experimental Sialadenitis, Anti-Salivary Gland Antibody, Sjögren's Syndrome, Immune Complex)

INTRODUCTION

Sjögren's syndrome (SJ S) is known as one of the rheumatic diseases, and is characterized by salivary and/or lachrymal gland destruction with mononuclear cell infiltrations (15). The similarity of such pathological changes to those of the thyroid gland in patients with Hashimoto's thyroiditis has been suggested (8). In addition, anti-salivary duct antibodies (3, 5, 7, 14) as well as cellular immune responses against salivary gland components (4, 11, 14, 16, 17) have been demonstrated in this syndrome. An autoimmune mechanism in the glandular destruction of these patients has been suggested for the above reasons although there is still no definite evidence.

The aim of the present study, therefore, was to investigate the possibility of experimental induction of sialadenitis in rats by heterologous anti-salivary gland antibodies.

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MATERIAL AND METHODS

(1) Fractionation of rat submaxillary gland:

Submaxillary glands were obtained from rats (Donryu strain, female), and stored at $-20^\circ C$ until use. The glands (8.2g) were homogenized in 40ml of Dulbecco's phosphate buffered saline (PBS) for 5 min, and then centrifuged at 8,000×g for 30 min. The supernatant was further centrifuged at 100,000×g for 60 min at 4°C, and the final supernatant (protein content: 3.8mg/ml) was used as a soluble fraction of the glands. The soluble fraction was dialysed against Tris-HCl buffer ($\mu = 0.05$, pH 8.0) supplemented with 1M NaCl, and fractionated by gel-filtration on a Sephadex-G200 column (2.5×100cm) with the same buffer. The first and second peaks of the two fractions identified were called Fraction I(FrI) and Fraction II(FrII), respectively (Fig. 1). Then each fraction was concentrated using polyvinylpyrrolidone after dialysis against distilled water. The protein content of the final fractions was 0.7mg/ml in FrI, and 3.2mg/ml in FrII. Each fraction was stored at $-20^\circ C$ before use.

A soluble fraction from rat liver was also separated in the same way.

![Fractionation of rat submaxillary gland by gel-filtration on a Sephadex-G200 column](image)

(2) Immunization with the fractionated submaxillary gland:

The FrI and II were diluted with an equal volume of PBS, and each diluted fraction was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories). Intradermal injections of each emulsion (1ml/rabbit/injection) were given to ten Japanese white rabbits (female, Wt; 2000g) three times at three-week intervals. The immunized
rabbit sera were obtained three weeks after the last injection.

(3) Transfer of the immunized rabbit sera: Three rats (Donryu strain, female, weighing 230—250g) were injected intraperitoneally with the sera (20ml/rat) of rabbits immunized with the FrI. Fourteen rats were also injected intraperitoneally with the sera (5—20ml/rat) of rabbits immunized with the FrII. All of the rats were sacrificed seven days after the serum transfer. Their organs such as the submaxillary gland, parotid gland, liver and kidney, were examined histopathologically by routine paraffin sections stained with hematoxylin-eosin. The submaxillary glands were also embedded, and frozen in acetone dry ice for the immunofluorescence study.

As control studies, sixteen rats were injected intraperitoneally with either 3.5% bovine serum albumin solution in PBS sterilized by a milipore filter, or normal rabbit serum (5—20ml/rat), and then sacrificed seven days after the injections.

(4) Ouchterlony immunodiffusion:
Immunodiffusion between the submaxillary gland fractions and the immunized rabbit sera was performed on a plate of 1% agarose in barbital buffer (pH 8.6, μ = 0.025) at 4°C.

(5) Immunofluorescence studies:
One ml of FITC-labelled anti-rabbit gamma globulin serum from goats (Behringwerke) was absorbed at 37°C for 120 min, and then at 4°C for 12 hours with an equal volume of rat liver acetone powder (Sigma) previously washed with PBS. Following the addition of 19ml of PBS, the supernatant was recovered by centrifugation at 2,500rpm for 10 min, and used as a FITC-labelled serum in further experiments.

(a) Direct immunofluorescence method: Cryosections (4μ) of the submaxillary glands from untreated rats or rats injected with immunized rabbit sera were fixed with 99.5% ethyl alcohol for 5 min. After washing three times for 15 min with PBS, each section was stained at 37°C for 30 min with the FITC-labelled serum and washed three times for 15 min with PBS. The sections were examined with a Zeiss Orthoflux fluorescent microscope after mounting with buffered glycerol.

(b) Indirect immunofluorescence method: Cryosections of the submaxillary gland from untreated rats were fixed and washed as described above. Next, the sections were incubated at 37°C for 30 min with rabbit sera absorbed previously with an equal volume of rat liver acetone powder, or with unabsorbed sera. Other sections were also incubated with PBS as a control study. After washing with PBS three times, all of the sections were stained with the FITC-labelled serum, and examined as described.

(6) Assay of antibody-dependent cell-mediated cytotoxicity (ADCC):
Fresh submaxillary gland from a rat was cut into 2mm³ sections by a sterile razor in RPMI 1640. Then, 50 of the tissue sections were washed with RPMI 1640, and incubated with 500μCi of Na₂⁵¹CrO₄ (1mCi/ml, 70mCi/<mg) at 37°C for 60 min in a 5%CO₂-incubator. The ⁵¹Cr-
labelled tissue sections were washed ten times with RPMI 1640.

Effector lymphocytes were isolated from heparinized venous blood of a normal rabbit on Ficoll-Hypaque gradients, and adjusted to $2 \times 10^6$/ml in RPMI 1640 containing 20% heat-inactivated fetal bovine serum.

The antisera obtained from rabbits immunized with the FrII, was heat-inactivated at 56°C for 30 min, and dilution of the antisera ($10^{-2}$ and $10^{-4}$) was performed by addition of the medium.

Each piece of the $^{51}$Cr-labelled tissue was mixed with 1.5 ml of the effector lymphocyte suspension in a sterile tube with a conical bottom. Then, 0.1 ml of the diluted antisera or the medium as a control study was added to the tissue-lymphocyte mixture. Following the incubation at 37°C for 5 hours in 5%CO$_2$-incubator, these mixtures were centrifuged, and 1 ml of the 1.6 ml supernatant as well as the remainder including the tissue section was counted in an automatic well-type gammacounter. All of the assays were performed in duplicate, and percent cytotoxicity was calculated as follows: 

$$\%^{51}\text{Cr-release} = \frac{\text{mean total supernatant count/mean total count}}{\text{total}} \times 100\%$$  

Maximum isotope release was estimated from cultures containing the $^{51}$Cr-labelled tissue and 1.6 ml of distilled water after three repeated freezings and thawings.

RESULTS

(1) Specificity and reactivity of the antisera from rabbits immunized with each fraction of rat submaxillary gland:

(a) Immunodiffusion: Two or three precipitating lines against the FrI, soluble fraction from rat liver or rat serum were identified in the anti-FrI serum. One of the two lines between the anti-FrI serum and the FrI was fused with the lines against rat liver or rat serum (Table 1). No precipitating line, however, was observed between the antisera and the FrII. All of the precipitating lines against FrI and rat liver were absorbed with rat liver acetone powder.

On the other hand, the anti-FrII serum did not react with rat liver or rat serum on immunodiffusion although one or two precipitating lines against the FrI or FrII were identified. The precipitating lines against the two fractions could not be absorbed completely with rat liver acetone powder.

<table>
<thead>
<tr>
<th>Table 1 Numbers of the precipitating lines on immunodiffusion</th>
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<tbody>
<tr>
<td>Antisera</td>
</tr>
<tr>
<td>Anti-FrI serum</td>
</tr>
<tr>
<td>not absorbed</td>
</tr>
<tr>
<td>absorbed*</td>
</tr>
<tr>
<td>Anti-FrII serum</td>
</tr>
<tr>
<td>not absorbed</td>
</tr>
<tr>
<td>absorbed*</td>
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</table>

* with rat liver acetone powder
(b) Indirect immunofluorescence method: Reactivity with normal rat submaxillary gland was demonstrated in each antiserum absorbed with rat liver acetone powder as well as untreated sera. Next, specific reactivity with cytoplasmic of the acinar cells and material in the secretory ducts was identified in the absorbed anti-FrlI serum (Photo 1 b). However, the absorbed anti-FrII serum reacted with acinar cell membrane or interstitial tissues, nuclear membrane of the secretory duct, and material in the duct (Photo 1 c).

(c) ADCC: %51Cr-release from the 51Cr-labelled tissue-lymphocyte cultures was 25.8% or 22.8% in the presence of 10^{-2} or 10^{-4} diluted anti-FrII serum, respectively. The %51Cr-release, however, was 11.9% in the control cultures (Table 2).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>%51Cr-release (%)</th>
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<tbody>
<tr>
<td>Serum dilution</td>
<td></td>
</tr>
<tr>
<td>10^{-2}</td>
<td>24.8</td>
</tr>
<tr>
<td>10^{-4}</td>
<td>22.8</td>
</tr>
<tr>
<td>Control without the serum</td>
<td>11.9</td>
</tr>
<tr>
<td>Maximum isotope release</td>
<td>49.9</td>
</tr>
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</table>

(2) Histopathological changes in rats transferred with the rabbit antisera:

In the submaxillary glands, scattered periductal cell infiltrations were demonstrated in ten out of fourteen rats transferred with anti-FrII sera, and in two of three rats transferred with the anti-FrI sera (Photo 2). The histological changes were similar in the two groups of rats, and the cell infiltrations were composed of mononuclear and polymorphonuclear cells including eosinophils. Many basophilic mast cells were also observed around the secretory ducts. Such cell infiltrations were not demonstrated in any of the submaxillary glands of rats transferred with normal rabbit serum or bovine serum albumin solution (Table 3).

In the parotid glands, however, small foci of such periductal cell infiltrations were observed in many rats transferred with the antisera. No such foci of cell infiltrations were detected in any of the rats transferred with bovine serum albumin although some of the rats transferred with normal rabbit serum showed similar changes in their parotid glands.

In the liver, similar small foci of cell infiltrations were also demonstrated around the bile ducts in many rats transferred with either the antisera or normal rabbit serum.

No glomerular lesions were demonstrated in the kidneys of rats although a few foci of similar cell infiltrations were detected around the renal tubulus in six of the fourteen rats transferred with the anti-FrII sera.

By the direct immunofluorescence method, granular deposits of rabbit gamma globulin were demonstrated in cytoplasm of the infiltrating cells as well as in the small vessels around the secretory ducts of the submaxillary gland (Photo 3).
Photo 2  Sialadenitis in the submaxillary gland of rats transferred with anti-FrH serum (HE staining). Periductal cell infiltrations were observed (a), and the cell infiltrations were composed of mononuclear and polymorphonuclear cells including eosinophils (b). Many basophilic mast cells were also observed around the secretory ducts.
**Photo 1** Indirect immunofluorescence method using rabbit antisera absorbed with rat liver acetone powder, and normal rat submaxillary gland (× 100)

The anti-FrI serum reacted with cytoplasm of the acinar cells and material in the secretory ducts (b). The anti-FrII serum reacted with acinar cell membrane, interstitial tissues, nuclear membrane of the ducts and material in the duct (c). Control was incubated with PBS, and then stained with the FITC-labelled serum (a).
Photo 3 Direct immunofluorescence method using the submaxillary gland of a rat transferred with anti-FrII serum (×200) Granular deposits of rabbit gamma globulin were observed in cytoplasm of the infiltrating cells and in the vessels around the duct (b). Such deposits were not demonstrated in the submaxillary gland of a normal rat (a).
Table 3  Cell infiltrations in rat organs

<table>
<thead>
<tr>
<th>Sera injected into the rats</th>
<th>Submaxillary gland</th>
<th>Parotid gland</th>
<th>Liver</th>
<th>Kidney</th>
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<tr>
<td>Anti-Frl serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 ml/rat</td>
<td>2/3</td>
<td>3/3</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Anti-FrlII serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 ml/rat</td>
<td>6/6</td>
<td>5/6</td>
<td>6/6</td>
<td>2/6</td>
</tr>
<tr>
<td>10 ml/rat</td>
<td>3/4</td>
<td>3/4</td>
<td>3/4</td>
<td>2/4</td>
</tr>
<tr>
<td>5 ml/rat</td>
<td>1/4</td>
<td>1/4</td>
<td>1/4</td>
<td>2/4</td>
</tr>
<tr>
<td>Total</td>
<td>10/14</td>
<td>9/14</td>
<td>10/14</td>
<td>6/14</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 ml/rat</td>
<td>0/4</td>
<td>2/4</td>
<td>2/4</td>
<td>0/4</td>
</tr>
<tr>
<td>10 ml/rat</td>
<td>0/2</td>
<td>1/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>5 ml/rat</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Total</td>
<td>0/8</td>
<td>3/8</td>
<td>2/8</td>
<td>0/8</td>
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<tr>
<td>Bovine serum albumin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 ml/rat</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
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<tr>
<td>10 ml/rat</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
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<tr>
<td>5 ml/rat</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
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</tr>
<tr>
<td>Total</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
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</tbody>
</table>

DISSCUSSION

The autoimmune mechanism of Sjögren’s syndrome (SjS) has been supported by evidence concerning humoral (3, 5, 7, 14) or cellular immune responses (4, 11, 14, 16, 17) against the salivary or lachrymal gland. In addition, experimental sialadenitis has been induced in animals immunized with homologous salivary gland or its components (9, 20, 21). Therefore, possible roles of cytotoxic lymphocytes and/or antibodies against the exocrine glands have been suggested in this syndrome although there has still been no definite evidence.

Concerning the roles of antibodies against the salivary components, Weill and Goldberg (1976) reported intracellular modification of acinar cells in the submaxillary gland in mice transferred with anti-mouse saliva antibodies from rabbits (19). These antibodies, however, did not induce inflammatory cell infiltration in the glands. In addition, when different antibodies recognizing different salivary components were injected, they all induced similar changes.

In the present study, therefore, we investigated the possibility of experimental induction of immune sialadenitis by heterologous antibodies against the salivary gland.

Specific reactivity with rat submaxillary gland was demonstrated in both of our two antisera obtained from rabbits immunized with FrI or FrII by immunodiffusion as well as the indirect immunofluorescence method although cross-reactivity of anti-FrI serum with rat liver and rat serum was shown. The anti-FrI serum absorbed previously with rat liver reacted with cytoplasm of the acinar cells and material in the secretory ducts. On the other hand, the anti-FrII serum reacted with acinar cell membrane or
interstitial tissues, nuclear membrane of the duct and the material in the ducts. ADCC activity against rat submaxillary gland was shown using anti-FrII serum in vitro.

Sialadenitis with periductal cell infiltrations in the submaxillary glands was induced in many rats transferred with the anti-FrI or FrII serum, and these cell infiltrations were composed of mononuclear and/or polymorphonuclear cells. Similar lesions, however, were also induced in the parotid gland, liver or kidney of these rats. Granular deposits of rabbit gamma globulin were detected in cytoplasma of the infiltrating cells or in the small vessels around the lesions by the direct immunofluorescence method.

In the above results, it was suggested that circulating immune complexes are be formed by non-organspecific antibodies contaminating the antisera, and then deposited in the organs. Several non-organ specific antibodies have been reported in sera of patients with SjS (6, 18), and circulating immune complexes have also been demonstrated in many of the patients (12). Additionally, similar salivary gland lesions to SjS have been demonstrated in many patients with other autoimmune diseases (1, 2, 10, 13). It was also suggested, therefore, that some of the lesions in patients with this syndrome might be induced by circulating immune complexes formed by such non-organ specific antibodies (12). The pathological significance of anti-salivary gland antibodies in vivo as an organ specific antibody could not be confirmed in the present study. Therefore, experiments using anti-salivary gland antisera absorbed with the other organs will be required for the further studies.

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REFERENCES


