Localization of Antibodies to Oral Microorganisms in Oral Mucosal and Submucosal Tissues of Healthy Rat

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Introduction

Inflamed gingiva of human contains immunoglobulins directed to several oral microorganisms\(^1 {\text{--}}^{4}\). The finding is consistent with the following hypothesis: as the inflammatory reaction of gingiva is regarded as the first step of the periodontitis\(^5\). Microorganisms invaded to gingiva encounter specific antibodies. Resulting immunocomplex may cause the activation of the classical complement pathway, which develops the inflammation followed by the chronic periodontitis.

In the healthy human serum or saliva natural occurring antibodies to *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus mitis*, *Lactobacillus acidophilus*\(^6\), *Leptotrichia buccalis*\(^7\) and *Bacteroides asaccharolyticus*\(^8\) are known. However the healthy human gingiva also contains low concentration of immunoglobulins\(^9 {\text{--}}^{11}\), specificities of which could not be known. Therefore the specific antibodies to oral microorganisms may also reside in the healthy gingival tissue.

In this paper we tried to clarify the localization of antibodies in oral mucosa using passive agglutination reaction with the extracts from various tissues.

Materials and Methods

Animals

The male rats of Wistar strain were fed for about 2 weeks and used for the study.

Collection of saliva and serum

The anesthetized rat was injected intraperitoneally with pilocarpine hydrochloride (1 mg/100g body weight) and the stimulated saliva was collected (about 0.5ml). Saliva from individual rat in the same volume was pooled and sodium azide was added up to 0.1% in concentration and the solution was kept at 4°C until used. The blood was obtained by cardiac puncture, coagulated at room temperature and kept at 4°C to separate serum. Then serum was prepared in the same manner as saliva.

Tissue extract

The various tissues were obtained from six rats after cardiac puncture. A section, 5 cm long, of small intestine containing one or two pieces of Peyer's patch was cut.
open. The tissues were washed twice with 50 ml of 0.02M potassium phosphate buffered (pH 7.2) saline (PBS) and weighed. The tissues were homogenized in a mortar with PBS (2ml/g tissue weight) and sea sand (1 ml/g tissue weight). Homogenates were squeezed through 2 layers of nylon gauze and the filtrates were concentrated by immersible CX-30 (Japan Milipore Ltd., Japan) or diluted by addition of PBS to adjust the concentration (2 ml/g tissue weight), and sodium azide was added, upto 0.1%, and kept at 4 C until used as the tissue extracts.

Bacterial sources

Freshly isolated strains from rats and stocked strains for generations were employed as sources of bacteria. The isolation procedure was as follows. After the anesthetization rats' teeth were rubbed with a swab, which was inoculated with glucose agar medium, glucose blood agar medium or tomato juice agar medium, and cells were cultured for 24 hr at 37 C. Stocked strains employed were Staphylococcus aureus 209p, Escherichia coli 0111 and Streptococcus mutans ingbritt which was provided by Dr. Takehara (Kyushu Dental College).

Preparation of cell wall fragments

Freshly isolated 7 strains were cultured in 400 ml of NIH thiglycolate medium, Baltimore Biological Laboratories (BBL), for 24—48 hr at 37 C. In the case of stocked strains cultivations were carried out in 400 ml of brain heart infusion broth (BBL). The cells were harvested by centrifugation and washed three times with PBS at 4 C. Following preparation were carried out at 4 C. The cells were suspended in PBS (5 ml/g wet weight) and destroyed in an ultra sonicator (model UR 200P, Tomy Seiko Co., Ltd., Tokyo) with equal weight of the glass beads (0.1mm diameter) at 100 W for 15 min. The ultra sonicates were centrifuged at 3,000xg for 15 min. The supernatants were centrifuged at 12,000xg for 30 min. Pellets (cell wall fragments) were resuspended in PBS to a proportion, 1 ml per 1 g.

Indirect heamagglutinable reaction

The cell wall fragments of each strain were conjugated to sheep blood red cells (SRBC) by glutaraldehyde treatment\textsuperscript{12). Resulting cell wall coated SRBC were used in 25 \textmu l of micro-titre system of indirect heamagglutinable reaction. In the reaction, the passive agglutinabilities of the extracts were tested, in which two fold of serially diluted extracts with 0.05M veronal buffered (pH 7.4) saline containing 0.1% gelatin were mixed with cell wall coated SRBC.

Single radial immunodiffusion

Immunoglobulins G, M, A and albumin contents in the tissue extracts, saliva and serum were measured by the single radial immunodiffusion. Anti-IgG and -IgM rabbit sera were purchased from Fujizoki Pharmaceutical Co., Ltd., Tokyo. Anti-IgA sheep, -albumin goat, -rabbit \textgamma globulin goat and -sheep \textgamma globulin sera were purchased from Cappel Laboratories Inc., PA, USA. In the case of the quantitative analysis of IgG and albumin a hundred \textmu l of anti-rat \textgamma-chain specific rabbit serum,
or albumin serum was mixed with 15 ml of PBS solution containing 0.6% agar, 0.6% agarose and 0.1% sodium azide at 55°C and applied on a glass plate (10 x 10 cm). In the case of the measurement of IgM or IgA, instead of former serum, 50 μl of anti-μ-chain specific or -α-chain specific sheep sera was utilized, respectively. Adequate volume of extracts were applied to the wells of the gel plate and incubated for 48 hr at 37°C under high humidity. Then the plate was dipped in the excess volume of PBS and incubated for 24 hr at 37°C. To measure the contents of IgG or albumin the plate was immersed in the distilled water for 2 hr, dried and stained by amido black 10B. In the case of the measurement of IgM or IgA, 4 μl of anti-rabbit or -sheep γ-globulin serum was applied in the wells of the plate as a secondary anti serum to impregnated one. The plate was washed with PBS for 24 hr, washed with distilled water, dried and stained. Immunoglobulin contents were measured as diameters of the ring of the resulting immuno-complex and expressed as proportions of the standard.

Protein contents

The proteins in tissue extracts, serum and saliva were precipitated by adding 10% trichloroacetic acid, and collected by centrifugation. The measurement of the quantities of the protein were performed by the method of Lowry et al (1951)\(^3\).

Results and discussion

Passive agglutinable reaction

The specific antibodies to microorganisms in the tissue extracts were examined by passive agglutinable reaction with various antigens coated SRBC. The tissue extracts exhibited the inconstant titres even using one sort of the coated SRBC. The various agglutinabilities meant the inequable distribution of the antibodies. This distribution may merely reflect the tissue contents of the immunoglobulins. Depending on this interpretation the distribution of titres comes to resemble in the reaction using any sorts of antigens, but dose not in Table 1. Therefore the titre might show the localization of the specific antibodies to oral microorganisms.

The haemagglutinable antibodies to the bacteria tested were frequently observed in oral mucosal and submucosal tissues such as gingiva, lower lip, cheek and hard palate and also in saliva and serum. And the antibodies to oral bacteria were localized in these oral mucosal and submucosal tissue also in healthy rat. On the other hand few agglutinability was observed in submandibular lymph node, submandibular gland, parotid gland, spleen and small intestine.

The difference between the reactions, using freshly isolated bacteria and stocked strains for many generations, even involved Enterobacteriaceae as E. coli, were not obvious. This may partly reflect the existence of the several related antigens in microorganisms, or provided from the habit of eating the excrements, which broadens the oral bacterial spectrum.
Table 1 Hemagglutination reactions with coated SRBC and various tissue extracts, serum and saliva.

<table>
<thead>
<tr>
<th>Tissue Extract</th>
<th>Isolated Oral Microorganisms</th>
<th>S. mutans</th>
<th>S. aureus</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. 1</td>
<td>No. 2</td>
<td>No. 3</td>
<td>No. 4</td>
</tr>
<tr>
<td>gingiva</td>
<td>3^2</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>lip</td>
<td>N^3</td>
<td>10</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>cheek</td>
<td>N</td>
<td>11</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>hard palate</td>
<td>4</td>
<td>9</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>tongue</td>
<td>N</td>
<td>12</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>submandibular gland</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>parotide gland</td>
<td>N</td>
<td>16</td>
<td>N</td>
<td>2</td>
</tr>
<tr>
<td>submandibular lymph node</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>2</td>
</tr>
<tr>
<td>spleen</td>
<td>N</td>
<td>17</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>small intestine</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>5</td>
</tr>
<tr>
<td>serum</td>
<td>N</td>
<td>2</td>
<td>N</td>
<td>3</td>
</tr>
<tr>
<td>saliva</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>9</td>
</tr>
</tbody>
</table>

1) No. 1—5: gram negative bacillus; No. 4: gram positive staphylococcus
2) Titres expressed as logarithmic scale
3) N: not detectable

Immunoglobulins contents in tissue extracts

Immunoglobulins contents in tissue extracts were expressed as the proportions to their serum or salivary contents (Table 2). The contents of albumin or the total proteins were rather steady values, and the extraction was performed in a similar efficiency. The contents of IgG or IgM in tissue extracts were low levels in the range from 0.031 to 0.067 fold or from 0.027 to 0.050 fold to serum, respectively. Immunoglobulin A was localized in oral mucosal and submucosal tissues and salivary glands, was too low to be detected in serum and its contents was expressed as proportions to saliva.

The antibodies to oral strain in these tissues may be attributed to salivary immunoglobulin, serum one or the one produced by local plasma cell. It was reported that IgA was localized in mucosal tissue which was always washed by saliva in which agglutinable antibodies were the class of IgA^6). However the contribution of saliva alone was not sufficient, because the titres compared to IgA contents in these tissues were much higher than that of saliva. As the concentration of the immunoglobulins in oral mucosal and submucosal tissues were lower than those in serum which revealed only a few titres, serum little concerned with the titres of these tissues. The plasma cells were sometimes appeared in healthy gingiva. Thus the localized antibodies seem to produced by local plasma cells and may originated a little from saliva. These tissues
Antibodies in oral mucosal and submucosal tissues (Inoue, et al.)

Table 2 Immunoglobulins, albumin and protein contents in tissue extracts, serum and saliva.

<table>
<thead>
<tr>
<th>Tissue Extracts</th>
<th>IgG Ratio to serum (%)</th>
<th>IgM Ratio to serum (%)</th>
<th>Albumin Ratio to serum (%)</th>
<th>IgA Ratio to saliva (%)</th>
<th>Protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gingiva</td>
<td>3.1</td>
<td>4.7</td>
<td>93.6</td>
<td>13.6</td>
<td>10.8</td>
</tr>
<tr>
<td>lip</td>
<td>3.3</td>
<td>3.6</td>
<td>178.0</td>
<td>2.4</td>
<td>9.2</td>
</tr>
<tr>
<td>cheek</td>
<td>4.4</td>
<td>5.0</td>
<td>176.8</td>
<td>7.6</td>
<td>12.0</td>
</tr>
<tr>
<td>hard palate</td>
<td>3.1</td>
<td>3.3</td>
<td>126.8</td>
<td>2.6</td>
<td>6.7</td>
</tr>
<tr>
<td>tongue</td>
<td>3.2</td>
<td>3.4</td>
<td>126.8</td>
<td>N*</td>
<td>13.5</td>
</tr>
<tr>
<td>submandibular gland</td>
<td>3.1</td>
<td>N</td>
<td>65.8</td>
<td>60.0</td>
<td>8.8</td>
</tr>
<tr>
<td>parotid gland</td>
<td>4.0</td>
<td>2.7</td>
<td>70.6</td>
<td>14.0</td>
<td>21.5</td>
</tr>
<tr>
<td>submandibular lymph node</td>
<td>6.7</td>
<td>N</td>
<td>96.4</td>
<td>N</td>
<td>14.8</td>
</tr>
<tr>
<td>spleen</td>
<td>4.4</td>
<td>4.5</td>
<td>40.2</td>
<td>N</td>
<td>18.2</td>
</tr>
<tr>
<td>small intestine</td>
<td>4.8</td>
<td>3.9</td>
<td>92.6</td>
<td>N</td>
<td>10.2</td>
</tr>
<tr>
<td>serum</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>N</td>
<td>82.5</td>
</tr>
<tr>
<td>saliva</td>
<td>2.0</td>
<td>N</td>
<td>1.8</td>
<td>100.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* N: not detectable

included scattered lymphoid aggregation or salivary glands scattered under mucosal tissue. And the plasma cells may be provided from these immunological tissues.

The characterization of the antibodies must clarify these attributions, but the preliminary examination to determine the class was unsuccessful.

Anyway the localization of antibodies in these oral tissues results in prompt responses to penetrating microorganisms.

Summary

The antibodies in various tissues extracts of healthy rat to oral bacteria were examined by passive agglutinatable reaction with coated sheep red blood cells by various antigens. Cell wall fragments of freshly isolated microorganisms from normal rat and stocked strains for many generations were used as antigens. The titres were higher in oral mucosal and submucosal tissues than in other tissues such as submandibular lymph node, parotid gland, submandibular gland, spleen and small intestine. Saliva and serum also showed the titres as high as oral mucosal and submucosal tissues. As immunoglobulins in saliva and serum were several as much as those in oral tissue extracts, saliva and serum immunoglobulins little attributed to the titres of these tissues.

Thus it was supported that the antibodies to oral bacteria were localized in oral mucosal and submucosal tissues also in healthy rat.
References


口腔常在菌に対する抗体の健全ラット口腔粘膜及び粘膜下組織への局在

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井 上 博 雅・内 山 長 司

健全ラット口腔組織中に口腔常在菌に対する抗体が存在するかどうかを、抗原を結合させたヒトジ赤血球細胞を用い、受身凝集反応により調べた。ラット口腔から新しく分離した菌及び保存菌数種から調製した細胞壁破片を抗原として用いた。口腔粘膜及び粘膜下組織抽出液に凝集抗体が認められ、顎下リンパ節、顎下腺、耳下腺、脾臓及び小腸等の組織抽出液では少なかったが認められない場合が多かった。唾液及び血清においても、口腔粘膜及び粘膜下組織抽出液とに同程度の凝集価が認められた。唾液及び血清中の免疫グロブリン含量はこれらの組織より数倍高いことから、これらの組織の凝集価に唾液や血清はほとんど寄与していないと考えられる。

以上のことから、健全なラットにおいて口腔粘膜及び粘膜下組織中に口腔常在菌に対する抗体が局在することが示唆された。