Immunological Cross-Reactivity of Oral Non-Streptococcal Bacteria with Mammalian Tissue

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The presence of cross-reacting antigens between oral non-streptococcal bacteria and human and monkey tissue was investigated by indirect immunofluorescence. Rabbit antisera, prepared against 22 oral bacteria, were incubated with cryostat-cut sections of heart, skeletal muscle, brain, spinal cord, liver, kidney, and skin. A cell wall antigen of Lactobacillus casei was shared with neuroglial brain cells, and an antigenic component of Propionibacterium acnes was shared with kidney glomeruli. Tissue-reactive antibodies were absorbed from the sera with preparations of both the homologous bacterium and tissue fractions.

Immunization against oral cariogenic bacteria has been proposed as a means for the prevention of dental caries (23). Before this approach can become a reality for humans, several parameters must be explored. One important consideration is whether such immunizations with oral bacterial antigens would elicit immunopathology in tissues. This might occur where common or cross-reactive (C-R) antigens are shared by both the bacteria and mammalian tissue. Antibodies induced by these C-R bacterial antigens may then react with tissue components, eliciting an inflammatory response. Rheumatic fever has sometimes been observed after immunization with type 3 streptococcal M protein and subsequent infections with group A streptococci (20). The incidence of rheumatic fever was significantly greater in the immunized subjects than in nonvaccinated controls.

Several of these C-R antigens have been detected in bacteria. The Forssman antigen which was originally found in some animal species such as guinea pigs and horses (31) cross-reacts with components of several bacteria, including Shigella dysenteriae, Streptococcus pneumoniae, and species of Salmonella (7, 26). Human blood group antigens of the ABO system also occur in a variety of gram-negative bacteria, including members of the genera Escherichia, Salmonella, Shigella, and Pseudomonas (26). Organ-specific C-R antigens are also shared by several bacterial species; for example, components of group A streptococci and various serotypes of S. mutans appear to cross-react immunologically with the mammalian heart (8, 28).

The purpose of this study was to investigate a selection of oral non-streptococcal microorganisms for antigens shared with human and monkey tissues.

MATERIALS AND METHODS

Bacteria. The bacteria used in this study are listed in Table 1. A number of cultures were kindly supplied by M. Gerencser, West Virginia University, Morgantown, W. Va.

Media and cultural conditions. Bacteria were grown on the chemically defined medium of Socransky (25) (1.5 liters in 2-liter Erlenmeyer flasks) or on difusate medium (24) containing brain heart infusion (Difco Laboratories), yeast extract, glucose, 5 μg of hemin per ml, and 0.5 μg of menadione per ml. For the growth of Lactobacillus casei, the chemically defined medium was supplemented with 0.5% citrate, 0.2% acetate, and 0.1% K2HPO4. Anaerobes were grown on preruced medium under an atmosphere of 5% CO2, 85% N2, and 10% H2. All cultures were incubated at 37°C. Growth was measured turbidimetrically, using a Klett-Summerson colorimeter (no. 66 filter). Cultures with optical density values between 100 and 250 Klett units were harvested by centrifugation at 10,000 × g for 15 min at 4°C. The cells were washed with 1 liter of ice-cold 0.15 M NaCl containing 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, (Tris-saline buffer; pH 7.5). The resulting pellet (0.5 to 2.5 g, wet weight) was suspended in 5 ml of distilled water and heated at 70°C for 30 min to inactivate possible autolytic enzymes.

The spent growth medium from the harvested culture was concentrated approximately 100-fold by ultrafiltration (PM-10 filter; Amicon Corp.) and heated to 70°C for 30 min. The concentrated extracellular material was then dialyzed against 2 liters of distilled water for 24 h with two bath changes and combined with the above cell preparation.

Preparation of antigens. The heated cell suspensions were disrupted by ultrasonic radiation by using a Bronwill Biosonic III (Bronwill Scientific Inc., Rochester, N.Y.). The cells were treated at 2 to 10°C until 60% breakage had occurred, as determined by optical density measurements and by phase-contrast microscopy. The broken cell preparations containing 4 to 20 mg of protein per ml were divided into small portions and stored at −20°C.
Bacterial cell fractionation. Exponential-growth-phase *L. casei* cells were harvested by centrifugation from 6 liters of culture medium. The cell pellets were washed twice with 1-liter quantities of Tris-saline buffer and resuspended in the same solution to a density of 100 mg of cells (weight) per ml. The bacteria were disrupted by shaking with glass beads in a Braun cell homogenizer at 4°C as described by Bleiweis et al. (2). The extent of cell breakage was between 90 and 95%. The lysed cell suspensions were separated into cell wall and cytoplasmic membrane fractions by differential centrifugation. All manipulations were conducted at 0 to 4°C, and the process consisted of the following.

(i) For the isolation of cell walls, the disrupted cell suspension was centrifuged at 10,000 × g for 30 min. The pellet, containing unbroken cells and cell wall fragments, was suspended in 250 ml of Tris-saline buffer and centrifuged at 1,000 × g for 15 min to remove unbroken cells. The supernatant fluid was centrifuged at 10,000 × g for 30 min to sediment the cell walls. The cell wall pellets were suspended in 250 ml of fresh Tris-saline buffer, using a motorized Teflon-glass tissue homogenizer, and were again subjected to a cycle of low- and high-speed centrifugation. This procedure was repeated (a total of 4 cycles) until all unbroken cells were removed. The resulting cell wall preparation was washed sequentially three times with 1 M NaCl and three times with distilled water and then heated in a boiling-water bath for 15 min (12). The final pellet was suspended in distilled water and stored at −20°C.

(ii) For the isolation of cytoplasmic membranes, the supernatant fluid from the initial centrifugation of the disrupted cell suspensions was centrifuged at 30,000 × g for 1 h. The supernatant fraction containing cytoplasmic components was collected and stored at −20°C. The pellet containing cytoplasmic membrane and some cell wall fragments was suspended in 200 ml of Tris-saline buffer and subjected to four cycles of differential centrifugation. Each cycle consisted of a low-speed centrifugation of 10,000 × g for 30 min to remove cell wall material, and a high-speed centrifugation of 30,000 × g for 1 h to sediment cell membranes. The resulting membrane preparation was washed three times with distilled water and stored at −20°C.

(iii) For the phenol-water extraction, polysaccharides and teichoic acids were extracted from isolated bacterial cell walls by treatment with hot 45% phenol by the method of Westphal and Jann (29).

Immunoization. The antigen suspensions for immunization were diluted in phosphate-buffered saline (pH 7.0) to 2 mg of protein per ml, and emulsions were prepared with equal volumes of complete Freund adjuvant (Grand Island Biological Co., Grand Island, N.Y.). Groups of three white male New Zealand rabbits received weekly intradermal injections with 0.1 ml of the appropriate vaccine. Immunization continued for 2 to 3 months until maximal antibody response to the bacterial antigens was achieved.

Brain homogenization and acetone extraction. A monkey brain (72 g, wet weight) was homogenized in approximately 4 volumes of 0.01 M KH₂PO₄-K₂HPO₄ buffer at pH 6.8 containing 0.25 M sucrose by the procedure of Koenig (11). A portion of the resulting suspension was used in absorption studies of rabbit anti-*L. casei* serum. Half of the brain homogenate was mixed rapidly with 9 volumes of cold acetone (4°C) and stirred for 30 min. The slurry was centrifuged at 4,000 × g for 10 min, and the supernatant fluid was removed. The residue was reextracted with a similar volume of acetone/water (9:1, vol/vol). The solvent fractions were combined and evaporated to dryness under a stream of nitrogen. The dried material was then suspended in 0.15 M NaCl, using ultrasonic radiation. The acetone-insoluble residue was treated in a similar manner.

**Assay for bacterial titers and cross-reacting antibodies.** Rabbit antisera to the bacteria were titrated by indirect immunofluorescence on washed, heat-fixed smears of the immunizing microorganism (22). Serial twofold dilutions of the antisera, in phosphate-buffered saline (pH 7) containing 4% bovine serum albumin, were incubated on the smears for 30 min followed by a 15-min wash in phosphate-buffered saline. Fluorescein-labeled goat anti-rabbit immunoglobulin G (IgG) was then incubated on the smears for 30 min, followed by a final PBS wash for 1 h. The goat anti-rabbit IgG conjugate was characterized by the methods of Beutner et al. (1). It had a molar fluorescein/protein ratio of 3.5, 16 U of antibody/ml, and was tested at 0.25 U of antibody/ml.

**Antisera to the bacteria were also tested by indirect**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Source*</th>
<th>Bacterial titerb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomyces bovis, serotype 1</td>
<td>WVU116</td>
<td>320</td>
</tr>
<tr>
<td>A. bovis, serotype 2</td>
<td>WVU292</td>
<td>160-320</td>
</tr>
<tr>
<td>A. eriksonii</td>
<td>WVU1507</td>
<td>320-640</td>
</tr>
<tr>
<td>A. israelii, serotype 1</td>
<td>WVU46</td>
<td>1,280</td>
</tr>
<tr>
<td>A. israelii, serotype 2</td>
<td>WVU428</td>
<td>1,280</td>
</tr>
<tr>
<td>A. naeslundii</td>
<td>WVU45</td>
<td>1,280</td>
</tr>
<tr>
<td>A. odontolyticus, serotype 1</td>
<td>ATCC 17929</td>
<td>320-2,560</td>
</tr>
<tr>
<td>A. odontolyticus, serotype 2</td>
<td>WVU482</td>
<td>640-1,280</td>
</tr>
<tr>
<td>A. tassiosus, serotype 2</td>
<td>WVU371</td>
<td>1,280</td>
</tr>
<tr>
<td>Bacterionema matruchotii</td>
<td>ATCC 14266</td>
<td>1,290-5,120</td>
</tr>
<tr>
<td>Bacteroides melanigenicus</td>
<td>ATCC 15032</td>
<td>640-1,280</td>
</tr>
<tr>
<td>Corynebacterium xerosis</td>
<td>ATCC 7094</td>
<td>160-640</td>
</tr>
<tr>
<td>Fusobacterium nucleatum</td>
<td>ATCC 10953</td>
<td>320-640</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>ATCC 393</td>
<td>640-1,280</td>
</tr>
<tr>
<td>Leptotrichia buccalis</td>
<td>ATCC 14201</td>
<td>1,280-2,560</td>
</tr>
<tr>
<td>Neisseria mucosa subsp. heidelbergensis</td>
<td>ATCC 25999</td>
<td>1,280</td>
</tr>
<tr>
<td>Nocardia saliense</td>
<td>ATCC 19426</td>
<td>1,280-5,120</td>
</tr>
<tr>
<td>Propionibacterium acnes, serotype 1</td>
<td>ATCC 6519</td>
<td>1,280</td>
</tr>
<tr>
<td>P. acnes</td>
<td>Fresh isolate</td>
<td>1,280-2,560</td>
</tr>
<tr>
<td>Rothia dentocariosa</td>
<td>ATCC 17931</td>
<td>320-640</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC 12000</td>
<td>10,240-20,480</td>
</tr>
<tr>
<td>Veillonella alcalescens</td>
<td>ATCC 17745</td>
<td>640</td>
</tr>
</tbody>
</table>

*ATCC, American Type Culture Collection number; WVU, West Virginia University number. All WVU cultures were supplied by Mary Gerencer.

b Titers from each of three rabbits immunized with the bacterium.

Table 1. Antiserum titers to microorganisms studied.
immunofluorescence (as described above) on rhesus monkey and human tissue. Each antiserum was tested on 4-μm-thick cryostat-cut sections of heart, kidney, skeletal muscle, smooth muscle, liver, brain, and skin. Sections were stored at −70°C for up to 2 weeks.

Where antiserum to bacteria reacted with mammalian tissue, specific absorptions were performed with preparations of bacteria and monkey tissues by incubating for 1 h at 37°C and then 18 h at 4°C. The sera were absorbed twice with an equal volume of antigen (Table 4).

Other assays. Total carbohydrate content was determined by the phenol sulfuric acid method of Dubois et al. (4). Protein was measured by the method of Lowry et al. (16), using crystalline bovine serum albumin as standard. Cell dry weight was determined with washed cells dried to constant weight.

RESULTS

The bacterial antibody titers assayed by indirect immunofluorescence are listed in Table 1. Titers ranged from 160 to 20,480 with most sera having titers ≥640. Serial twofold dilutions of preimmune and immune sera, beginning at 1:10, were also reacted with the following human and monkey tissues: heart, kidney, skeletal muscle, smooth muscle, liver, brain, and skin. Among the immune sera evaluated, antiserum to two bacteria reacted with mammalian tissues, whereas the preimmune sera from the same rabbits were nonreactive.

Antiserum to L. casei reacted with neuroglia cells and their processes in human and monkey nervous tissue (Fig. 1 and 2). Anti-L. casei serum with a bacterial titer of 1,280 gave titers of 40 on monkey brain and 10 on human brain (Table 2). The extent of this reactivity within the central nervous system of monkeys was examined. Titers of 40 to neuroglia cell processes were observed in the cerebral cortex, cerebellum, corpus callosum, and gray matter of the spinal cord. Lower titers of 10–20 were observed in the semiovale and white matter of the spinal cord.

The specificity of this reactivity was examined by absorption of antil. casei serum with L. casei cells and monkey brain fractions. The biochemical characteristics of antigens used for absorption are listed in Table 3. Antiserum to L. casei was absorbed with L. casei preparations, including ultrasonically disrupted bacterial cells, cell membranes, cell walls, and fractions from a phenol-water extraction of cell walls (Table 4). They were also absorbed with a monkey brain homogenate and fractions of acetone-extracted monkey brain.

Two absorptions of anti-L. casei serum with disrupted bacteria reduced the bacterial titers from 1,280 to 40 and reactivity with the neuroglia cell processes from 40 to 20. Absorption of anti-L. casei with the interphase material from the phenol-water extraction reduced the titer to neuroglia cell processes from 40 to 10 or less, whereas the materials in the phenol and water phases had little or no effect. Absorption with the cell wall preparations also reduced the titer to the neuroglial cell processes to 5 or less (Fig. 1).

Antiserum to a freshly isolated strain of Propionibacterium acnes reacted with the glomeruli but not the capsule of human and monkey kidney (Fig. 3). An antiserum with a titer of 1,280 to the immunizing strain of P. acnes gave titers of 160 and 16 on human and monkey glomeruli, respectively (Table 5). To examine the specificity of this cross reaction, antiserum to P. acnes was absorbed with an equal volume of the preparation of P. acnes used for immunization. This single absorption with the bacteria reduced the bacterial titer from 1,280 to 10, while also reducing the human glomerular titer from 160 to 10 (Fig. 3) and the monkey glomerular titer from 16 to 4.

DISCUSSION

Rabbit antiserum to L. casei were found to react with neuroglial cells in the brain and spinal cord, whereas antiserum to a strain of P. acnes reacted with the glomeruli of the kidney. All immune and preimmune sera reacted with the stratum corneum of skin. The intensity, titer, and pattern of stratum corneum reactivity were identical and therefore, were not considered to be a result of immunization.

C-R antigens of bacteria reacting with brain have been reported in Streptococcus pyogenes type 24 (10) and type 6 (9). Antisera to type 24 reacted with the limiting membrane and its fibers, ependymal fibers and neuroglia of mouse brain. Ultrastructural studies of the glial reactivity suggest that antiserum to S. pyogenes type 24 cross-reacts with fibrous astrocytes (3).

L. casei shares C-R antigens with the brain which appear to be organ specific. Antiserum to L. casei did not react with the other tissues, including smooth muscle, striated muscle, heart, liver, kidney, or skin. Moreover, the C-R antigens shared by brain and L. casei may be different from those shared with S. pyogenes. Anti-L. casei sera demonstrated frequent high-titer staining of neuroglia cell processes in the gray matter of the spinal cord and less intense staining of the white matter where fibrous astrocytes chiefly occur. Anti-S. pyogenes sera, however, have been shown to react most strongly with fibrous astrocytes. Confirmation of the glial cell type reactive with anti-L. casei sera will require ultrastructural studies.

The specificity of the brain reactivity with
Fig. 1. Indirect immunofluorescence micrograph of monkey cerebral cortex treated with rabbit anti-L. casei serum (1:10). (A) Immune serum-treated tissue. Glial processes or fibers are stained. (B) Tissue treated with immune serum absorbed with L. casei cell walls. (×400).

Fig. 2. Indirect immunofluorescence micrograph of the grey matter of monkey spinal cord after incubation with rabbit anti-L. casei serum (1:10). The processes and cytoplasm of a glial cell are stained, as are other processes. ×400.

anti-L. casei sera was confirmed by absorption studies. The brain reactivity could be reduced or eliminated by absorption with either L. casei or monkey brain preparations. The reproducibility of the indirect immunofluorescent test was such that titers sometimes varied by plus or minus one doubling dilution on repetitive testing. Therefore, a onefold dilution decrease in titer after absorption was not considered significant. The interphase material from the phenol-water extraction of L. casei and the cell wall preparations significantly reduced the titer to neuroglial cells. However, absorption with cell material in the phenol and water phases of the phenol-water extraction or the cell membrane preparation did not significantly change the titer to neuroglia. This suggests that the C-R antigen(s) of L. casei is a component of the cell wall and may not consist of polysaccharide or teichoic acids, components that are solubilized by phenol-water extraction (14, 27, 30).
Removal of anti-\textit{L. casei} serum cross-reactivity with glial cells was also achieved by absorption with monkey brain homogenate and the acetone-soluble fractions of monkey brain but not with the acetone-insoluble residue. The C-R antigen(s) in the neuroglia appear to be associated with lipid components since acetone/water (9:1, vol/vol) extraction removes neutral lipids and many phospholipids from mammalian membranes (15).

A freshly isolated strain of \textit{P. acnes} contained antigens that cross-reacted with the glomerulus of the kidney. These C-R antigens were apparently either strain specific or lost by \textit{P. acnes} during long term subculture, since they were only observed in antisera prepared to the freshly isolated strain and not in antisera to the American Type Culture Collection-derived strain of \textit{P. acnes}. It is generally recognized that prolonged in vitro cultivation increases the risk of antigenic variation via loss of plasmids, selection of defective mutants, or gene repression mechanisms. To evaluate this possibility, further studies of this kind should include a comparison of fresh clinical isolates and established stock strains of bacteria.

The C-R antigens shared by \textit{P. acnes} and mammalian tissue also exhibited organ specificity and some limited species specificity. Antisera to \textit{P. acnes} reacted with kidney but not with smooth muscle, striated muscle, heart, liver, or skin. In addition, it appears that human glomeruli may share multiple antigens with \textit{P. acnes}, some of which are not seen in monkey kidney, since anti-\textit{P. acnes} sera reacted more strongly with human glomeruli (titer 160) than it did with monkey glomeruli (titer 16). The specificity of the C-R antigens shared by \textit{P. acnes} and the glomeruli was revealed by absorption studies. Absorption of anti-\textit{P. acnes} serum with bacteria not only reduced the immunofluorescence titer to \textit{P. acnes} cells from 1,280 to 10, but also reduced the titer to human glomeruli from 160 to 10.

C-R antigens of bacteria reacting with kidney have also been observed in "nephritogenic" strains of \textit{Streptococcus pyogenes}, type 12 (18, 19), \textit{Escherichia coli} 02, 014, and 022 (6), and common enterobacterial antigen (5).

Although several C-R antigens have been identified in bacteria and mammalian tissue, few appear to induce autoimmune reactions (17). The C-R antigens of \textit{S. pyogenes} that are shared with heart tissue, however, appear capable of inducing tissue-reactive antibodies which may elicit myocardial inflammation (17). Sera obtained from patients with rheumatic fever have antibodies to heart tissue that can be removed by absorption with cell walls of \textit{S. pyogenes}. The removal of heart-reactive antibody has been attributed to the presence of a C-R antigen in streptococci (8, 17). This interpretation, however, must be viewed with some caution, since cell walls of some streptococci (groups A, C, and G) have the capacity to bind to the Fc region of IgM molecules (13, 21). Such an Fc receptor does not appear to be present in \textit{L. casei} or \textit{P. acnes} cells since, in this study, these bacteria did not

\begin{table}
\centering
\caption{Indirect immunofluorescent titers with anti-\textit{L. casei} serum}
\begin{tabular}{llll}
\hline
Substrate & Preimmune serum & Immune serum & \\
\hline
\textit{L. casei} & 10 & 1,280 & \\
Human brain & <10 & 10 & \\
Monkey brain & <5 & 40 & \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Antigens for absorption of antisera to \textit{L. casei}}
\begin{tabular}{llll}
\hline
Antigen source & Composition (mg/ml) & Protein & Carbohydrate & \\
\hline
\textit{L. casei} & & & & \\
Whole cell homogenate & 1 & 0.3 & \\
Cell membrane & 2.2 & 0.8 & \\
Cell wall & 2.4 & 2.0 & \\
Phenol-water extraction & & & & \\
Phenol phase & 0.02 & 0.05 & \\
Water phase & 0.9 & 4.8 & \\
Insoluble residue & 3.9 & 4.0 & \\
Monkey brain & Homogenate & 20 & \\
Acetone-soluble extract & <0.01 & \\
Acetone-insoluble residue & 2.8 & \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Absorption of anti-\textit{L. casei} serum}
\begin{tabular}{llll}
\hline
Absorption antigen & Doubling dilution change in titer vs: & \\
& \textit{L. casei} & Monkey brain & \\
\hline
\textit{L. casei} & & & \\
Sonic extract & -5 & -1 & \\
Phenol extraction & & & \\
Phenol phase & -3 & 0 & \\
Water phase & ND & 0 to -1 & \\
Interphase & \textit{ge} & \textit{ge} & \\
Cell wall & \textit{ge} & -3 & \\
Cell membrane & \textit{ge} & 0 to -1 & \\
\hline
Monkey brain & Homogenate & 0 & -2 & \\
Acetone extract & -1 & -1 to -2 & \\
Acetone residue & 0 & 0 & \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} Value indicates doubling dilution change in serum titer after two absorptions with the indicated antigen.

\textsuperscript{b} ND, Not determined.
bind significant quantities of IgG from normal rabbit sera as determined by indirect immunofluorescence. The C-R antigens of *L. casei* and *P. acnes* detected in the present study may be capable of inducing tissue-reactive antibodies. In considering immunization against dental caries using oral bacteria, the potential for C-R antigens must be explored for each strain of microorganism. When such antigens are found, immunization would best be pursued with purified bacterial preparations lacking the C-R antigens.

**ACKNOWLEDGMENTS**

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We thank Eileen A. McCafferty and Deborah G. Curtiss for their excellent technical assistance.

**LITERATURE CITED**


**TABLE 5. Indirect immunofluorescence tests with anti-*P. acnes* serum**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Titer</th>
<th>Absorption: change in titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. acnes</em></td>
<td>10</td>
<td>1,280</td>
</tr>
<tr>
<td>Human kidney</td>
<td>&lt;10</td>
<td>160</td>
</tr>
<tr>
<td>Monkey kidney</td>
<td>&lt;2</td>
<td>16</td>
</tr>
</tbody>
</table>

* Doubling dilution change in titer after two absorptions of anti- *P. acnes* with disrupted *P. acnes* (corrected for absorption dilution).

**FIG. 3. Indirect immunofluorescence micrograph of human kidney treated with rabbit anti-*P. acnes* serum (1:10) (A) Immune serum. Staining is seen in the glomerulus. (B) Immune serum absorbed with disrupted *P. acnes* cells. ×400.


