Antibodies to Acholeplasma laidlawii Membrane Lipids in Normal Guinea Pig Serum

ISOLDE DÖRNER,† HELMUT BRUNNER,† HANS-GERD SCHIEFER, MICHAEL LOOS, AND HANS-JOBST WELLENIEK

Institut für Medizinische Mikrobiologie, Justus Liebig Universität, D-6300 Giessen, and Institut für Medizinische Mikrobiologie, Johannes Gutenberg Universität, D-6500 Mainz, Federal Republic of Germany

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Acholeplasma laidlawii is killed and lysed by fresh normal guinea pig serum (GPS) without additional antibodies. Prior incubation of GPS with whole A. laidlawii organisms abolishes the killing activity of GPS. In the present study it was demonstrated that antibodies are present in normal GPS. The classical pathway, not the alternative pathway, of the complement sequence was activated by these antibodies in fresh normal GPS. The antibodies in GPS belong to the IgG class of immunoglobulins. They are directed predominantly against the membrane phospholipids of A. laidlawii. These antibodies may be induced either by natural infection of guinea pigs with A. laidlawii or by antigenic determinants of other microorganisms or food antigens.

In recent years it has been clearly demonstrated that some mycoplasmas are killed and lysed by the combined action of antibodies and complement (1, 6, 8, 12, 15, 20, 24, 25, 34). Fresh normal guinea pig serum (GPS) has been most frequently used as the source of complement in these experiments. In studies on immune lysis of Mycoplasma pneumoniae it was noted that GPS by itself, without addition of antibodies, reduced the number of viable organisms. As had been reported by Gale and Kenny (15), Sethi and Teschner (34), and from our laboratory (8), removal of the growth-inhibitory substances from GPS by absorption with killed M. pneumoniae was not possible. Nevertheless, the possibility that small amounts of antibodies to M. pneumoniae, not detectable by our methods, were present in normal GPS could not be excluded.

It has been recently shown by Bredt and Bitter-Suermann (4) that M. pneumoniae can activate the guinea pig complement system via the alternative pathway. This activation results in lysis of the organisms. Efficient killing requires, in addition, the entire complement sequence. It was also shown that M. pneumoniae can interact directly with C1 in the absence of detectable amounts of antibodies (5).

We have previously demonstrated that a decrease in viability of Acholeplasma laidlawii occurs when the organisms are incubated with fresh GPS (11). In this publication, data are presented indicating that antibodies of the immunoglobulin G (IgG) class can be detected in normal GPS. These antibodies are directed against the membrane lipids of the organisms and activate preferentially the classical pathway of complement.

(These results were presented in a preliminary form at the Annual Meeting of the American Society for Microbiology, New York, 1975, and at the Arbeitstagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Mainz, West Germany, 1976).

MATERIALS AND METHODS

Organisms and growth media. A. laidlawii, oral strain, and M. pneumoniae, strain PI 1428, originally obtained from R. M. Chanock, Bethesda, Md., were used. A. laidlawii had been subcultured several times on artificial medium. M. pneumoniae was used in its sixth passage on growth medium (9, 11).

The medium for A. laidlawii consisted of 350 ml of PPLO broth (Difco Laboratories, Detroit, Mich.) supplemented with 5 ml of PPLO serum fraction (Difco), 50 ml of 1% yeast extract (Oxoid Ltd., London), 10 ml of 50% glucose, 10 ml of 0.1% phenol red, 12.5 ml of 2% thallium acetate, and 1,000 U of penicillin G per ml. The pH was adjusted to 8.2 with 1 N sodium hydroxide. The growth medium for M. pneumoniae consisted of 350 ml of PPLO broth; 100 ml of agamma horse serum (Flow Laboratories, Inc., Rockville, Md.) that had been inactivated at 56°C for 30 min, 50 ml of 25% yeast extract (Flow), 10 ml of 50% glucose, 10 ml of 0.1% phenol red, 12.5 ml of 2% thallium acetate, and 1,000 U of penicillin G per ml.

Culture conditions. For killing experiments, A. laidlawii was grown at 37°C in screw-capped bottles containing 500 ml of broth medium and was harvested...
after 36 h when the medium started to change color. To obtain large quantities of A. laidlawii, the organisms were grown in 10- to 20-liter batches. After incubation at 37°C for 36 h the organisms were harvested by centrifugation at 36,000 × g for 20 min.

The pellets were washed three times in 0.25 M NaCl. M. pneumoniae was grown on the glass surfaces of 5-liter Povitsky bottles containing 1 liter of medium. The organisms were harvested after incubation at 37°C for 5 to 6 days. The medium was decanted, and the sheet of organisms adhering to the glass was scraped off in v% of the initial volume of the medium.

The organism suspensions were distributed in small quantities in glass ampoules, which were sealed, shell-frozen in a Dry Ice-alcohol bath, and kept at −85°C until used. Once thawed, they were not refrozen.

**Buffers.** The reagents were made up either in Veronal-buffered saline, pH 7.4, or in 0.01 M N-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid (TES)-buffered saline, pH 7.2. When necessary, the buffers were supplemented with 0.15 mM CaCl₂, 0.5 mM MgCl₂, and 5 mM ethylenediaminetetraacetate (EDTA) (21). For some experiments phosphate (0.002 M)-buffered saline (PBS), pH 7.2, was used as the diluent.

**Isolation of cell membranes.** Cell membranes were isolated after lysis of the organism by osmotic shock (27). For osmotic lysis (27), the suspension of A. laidlawii was centrifuged at 36,000 × g for 30 min. The sedimented cells from 10 liters of growth medium were washed five times in saline, resuspended in 250 ml of deionized water, and incubated at 37°C for 15 min to lyse the cells. After incubation, the mycoplasma suspension was centrifuged at 8,000 × g for 5 min to remove clumps of unbroken cells. The supernatant fluid was then centrifuged at 36,000 × g for 30 min to collect the membranes.

**Separation of A. laidlawii membrane proteins from membrane lipids.** The method of Fleischer and Fleischer (13) was used to separate membrane proteins from membrane lipids. The washed membranes were suspended in deionized water (10 mg of protein per ml) and added to nine times the volume of acetone. One drop of 28% NH₄OH per 50 ml of the suspension was added. The mixture was kept at room temperature for 30 min and then centrifuged at 25,000 × g for 30 min. The sediment was resuspended in deionized water, again extracted with acetone-NH₄OH, and centrifuged. This second sediment contained the lipid-depleted material henceforth referred to as membrane protein. The two supernatant fluids contained the lipids.

**Separation of membrane lipids.** The aqueous acetone extract of A. laidlawii membrane lipids was concentrated in an atmosphere of nitrogen and fractionated into neutral lipids, glycolipids, and phospholipids by column chromatography on silicic acid, as described previously (11).

**Thin-layer chromatography.** The fractions were studied by thin-layer chromatography (solvent system: chloroform-methanol-water [70:25:5]) (11).

**GPS and C4-deficient GPS.** Thirty guinea pigs were sacrificed, and their blood was collected by cardiac puncture. The serum was separated after clotting overnight at 4°C, distributed in small quantities, and stored at −85°C. A single pool of GPS was used for all experiments. Sera of individual guinea pigs of different ages were also collected, and each serum was kept separately and stored at −85°C. In addition, pools of fresh frozen C4-deficient GPS were used (14).

**Fractionation of GPS.** A 20-ml amount of GPS was applied to a column (5 by 90 cm) of Sephadex G-200 and eluted with PBS supplemented with 5 mM EDTA. Fractions of 10 ml were collected, and 0.1 M MgCl₂ and 0.3 M CaCl₂ at a final dilution of 1:100 were added. The fractions comprising an elution maximum were combined and concentrated 40-fold by pressure dialysis (Amicon Corp., Lexington, Mass.).

The fractions containing predominantly 75 immunoglobulins were applied to a sucrose gradient for further purification. Centrifuge tubes were filled with 0.9 ml each of 26, 22, 18, 14, and 10% sucrose in 0.05 M PBS. The gradient was incubated for 18 h at 4°C for equilibration. A 0.5-ml amount of the second elution peak from Sephadex G-200 diluted 1:2 in PBS was applied to the gradient and centrifuged at 35,000 × g for 15 h. The 75 and 19S fractions were dialyzed against saline for 12 h.

**Titration of complement components.** The methods for the preparation of sheep erythrocytes, cell intermediates (EA, EAC1, EAC14, EAC142), and antisera are given by Rapp and Borsos (26). The titration of C1, C4, and C2 with guinea pig complement plus EDTA as the source of late-acting components was performed as described by Rapp and Borsos (26). Complement plus EDTA was obtained by adding 5 mM EDTA to GPS.

**Test for consumption of complement components.** To determine consumption of individual complement components during incubation of A. laidlawii with GPS, equal volumes of a concentrated suspension of A. laidlawii in TES-buffered saline and GPS were incubated for 1 h at 37°C. The suspension was centrifuged at 36,000 × g for 30 min, and the supernatant was tested for consumption of complement components (50% hemolytic complement, C1, C4, C2, and C3) in the hemolytic system and compared to untreated controls (2, 3, 26).

**Preparation of lipid antigens.** The various lipid antigens (total lipids, neutral lipids, glycolipids, and phospholipids) were prepared by mixing 0.5 mg of the lipid with 5 mg of phosphatidylcholine (from egg yolk) in chloroform. The solvent was evaporated to dryness under nitrogen. After the lipids were redissolved in 0.1 ml of ethanol, 0.9 ml of Veronal-buffered saline, preheated to 60°C, was added and mixed well.

**Antiserum.** Hyperimmune rabbit antiserum to whole sheep erythrocytes was obtained from Behringwerke, A. G., Marburg/Lahn, West Germany. Rabbit antiserum to guinea pig IgG and IgM were obtained from Byk-Mallinckrodt, Dietzenbach-Steinberg, West Germany.

**Preparation of zymosan.** A 100-g amount of bakers' yeast was suspended in 2 liters of PBS and autoclaved for 30 min. The suspension was centrifuged at 5,000 × g for 10 min, and the sediment was washed twice with PBS. The sediment was resuspended in 200 ml of PBS, autoclaved, and stored at 4°C.

**Mycoplasmacladic test.** The organisms were filtered through a prewashed 450-nm pore-size mem-
brane filter (Millipore Corp., Bedford, Mass.) to obtain a homogeneous suspension of predominantly single organisms (7). To determine the reduction in viability, various concentrations of GPS were added to 0.1 ml of the suspension of filtered organisms (containing approximately $1 \times 10^9$ to $5 \times 10^7$ colony-forming units per ml). TES-buffered saline, pH 7.2, supplemented with 0.15 mM Ca$^{2+}$ and 0.5 mM Mg$^{2+}$ was used as the diluent. After incubation for 1 h at 37°C, 0.1 ml of each mixture was withdrawn and diluted 1:100 in ice-cold TES-buffered saline to stop the reaction. Controls containing buffer alone were included. After stopping the reaction, two further 10-fold dilutions of the organism suspension were prepared in PPLO broth without additives, and 0.1-ml quantities of each dilution were inoculated in triplicate onto agar medium. After incubation at 37°C, colonies were counted.

GPS preparations tested in the mycoplasmacidal assay. Besides GPS, GPS preincubated for 1 h at 4°C with either A. laidlawii, A. laidlawii plus 5 mM EDTA, 5 mM EDTA alone, or zymosan alone was tested for cidal activity on A. laidlawii. A. laidlawii organisms were removed from the GPS by centrifugation at 5,000 x g for 10 min. Serum preincubated with EDTA was recalculated as described above.

Immune adherence assay. Immune adherence was tested using microtiter equipment (22). Twofold dilutions of serum and serum fractions obtained by gel filtration on Sephadex G-200 were performed in 0.025 ml of TES-buffered saline containing 0.15 mM Ca$^{2+}$. A 0.025-ml amount of a concentrated suspension of A. laidlawii in TES-buffered saline (containing about $10^9$ colony-forming units per ml) was added. After incubation at 4°C for 30 min, 0.025 ml of GPS preincubated with A. laidlawii organisms in the cold and diluted 1:10 in TES was added. The mixture was further incubated at 37°C for 15 min. A 0.025-ml amount of washed human type O, Rh-positive red blood cells in TES-buffered saline, containing $5 \times 10^8$ cells/ml, was added. The plates were examined after incubation at 37°C for 30 min. The serum fractions from gel filtration were tested again after heat inactivation (56°C, 30 min).

Double-diffusion test in agar. The test was performed according to Ouchterlony (23). Purified agar (4%, wt/vol) (Behringwerke) was mixed with 0.15 M PBS, pH 7.1, and diluted 1:4 in deionized water supplemented with Merthiolate (1:10,000). The mixture was heated to 100°C. Microscope slides were covered with melted agar. Six peripheral holes and one central hole of 2-mm diameters were cut into the agar. Five microliters of the 7S fraction obtained from the sucrose gradient were filled into the central hole. Five microliters of anti-IgG and anti-IgM (Nordic Pharmaceuticals, Ltd., Quebec, Canada) in various dilutions were filled into the peripheral holes. The slides were kept at room temperature in a moist chamber. Optimal precipitation lines were detected after 48 h of incubation.

RESULTS

Effect of GPS on viability of A. laidlawii. Fresh GPS killed A. laidlawii when tested at a dilution of 1:32 or lower (Fig. 1). A decrease in viability was not observed when A. laidlawii was mixed with GPS that had been preincubated at 4°C with organisms, indicating that the activity could be removed by absorption with A. laidlawii. In a previous publication we have demonstrated that the killing activity of fresh absorbed GPS could be restored by the addition of heat-inactivated (56°C, 30 min) rabbit antiserum to A. laidlawii (11). This indicates that the complement activity of the fresh GPS absorbed in the cold is retained during the absorption procedure.

The killing effect of fresh unabsorbed GPS on A. laidlawii can be explained either by the activation of the alternative pathway of complement by A. laidlawii or by the presence of antibodies to A. laidlawii in normal GPS.

To test these possibilities, the killing effect of GPS preincubated with A. laidlawii in the presence of EDTA or with zymosan was tested (Fig. 1). As can be seen, the mycoplasmacidal activity of the serum was abolished when it had been preincubated with A. laidlawii in the presence of EDTA, but not when it was preincubated with zymosan. This suggests that another factor(s) besides the complement system is essential for the mycoplasmacidal effect of fresh GPS. This factor(s) may be antibodies.

Characterization of serum factors involved in the mycoplasmacidal effect of GPS. To find out in which fraction(s) of the serum the mycoplasmacidal activity can be found, GPS was applied to a column of Sepha-
Optical density (OD), and individual fractions were tested for mycoplasma-specific immune adherence. Positive reactions could only be detected in the second peak eluted from the column (Fig. 2). This peak contains, predominantly, IgG. Heat inactivation (56°C, 30 min) of the fractions was not accompanied by a decrease in immune adherence titer.

To further demonstrate that IgG was involved, immune adherence-positive fractions were applied to a sucrose gradient. The 7S fractions only were positive in immune adherence (titer of 1:64). No activity could be detected in the 19S fraction (titer of <1:2). Double-immunodiffusion tests in agar with the 7S fraction, using rabbit anti-guinea pig IgG and IgM, demonstrated the identity of the 7S fraction with IgG.

Effect of *A. laidlawii* and its different membrane components on complement activity of GPS. Reduction of 50% hemolytic complement, C1, C4, and C3 was determined in GPS after incubation with whole *A. laidlawii* cells, *A. laidlawii* membrane protein, and *A. laidlawii* membrane lipids. A significant reduction in 50% hemolytic complement, C1, C4, C2, and C3 activity was observed with whole *A. laidlawii* cells and *A. laidlawii* membrane lipids, but not with *A. laidlawii* membrane protein (Table 1). Maximal reduction of complement activity was obtained with the phospholipid fraction, whereas neutral lipids and glycolipids did not show a significant effect. These findings further indicate that the classical pathway of complement is activated. In addition, it is demonstrated that the antibodies in normal GPS are directed against the membrane phospholipids of the organisms.

When serum of guinea pigs deficient in C4 was exposed to *A. laidlawii* cells, *A. laidlawii* membrane protein, and *A. laidlawii* membrane lipids, a significant reduction was observed in C1 activity only (Table 2). This also indicates that the classical complement pathway is activated in fresh GPS.

Antibodies to *A. laidlawii* in normal GPS from animals of various ages. The data so far were obtained on GPS pooled from 30 adult animals. If the antibodies to *A. laidlawii* are acquired by exposure to a cross-reactive lipid antigen, one would assume that the titers of these antibodies should increase with age. This hypothesis was tested by determination of immune adherence titers in GPS from animals of various ages. Titers of antibodies to *A. laidlawii* increased with age (Table 3), but even at the age of 1 week small amounts of antibodies to *A. laidlawii* were detectable.

Specificity of antibodies to *A. laidlawii*

### Table 1. Effect of *A. laidlawii* and its various membrane components on complement activity of normal GPS

<table>
<thead>
<tr>
<th>Complement activity</th>
<th>Reduction (%) at 37°C in <em>A. laidlawii</em>:</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cells</td>
</tr>
<tr>
<td>CH50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C1</td>
<td>96.9</td>
<td>98.6</td>
</tr>
<tr>
<td>C4</td>
<td>95.7</td>
<td>99.3</td>
</tr>
<tr>
<td>C2</td>
<td>70.3</td>
<td>61.9</td>
</tr>
<tr>
<td>C3</td>
<td>86.8</td>
<td>71.2</td>
</tr>
</tbody>
</table>

* After incubation of equal volumes of either *A. laidlawii* cells (0.4 mg/ml), *A. laidlawii* membrane lipids (0.4 mg/ml), or *A. laidlawii* membrane protein (0.5 mg/ml) and of normal guinea pig serum for 30 min at 37°C, 50% hemolytic complement (CH50), C1, C4, C2, and C3 were determined and compared to buffer controls.

### Table 2. Effect of *A. laidlawii* and its various membrane components on complement activity of C4-deficient GPS (C4dGPS)

<table>
<thead>
<tr>
<th>Complement activity of C4dGPS</th>
<th>Reduction (%) at 37°C in <em>A. laidlawii</em>:</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cells</td>
</tr>
<tr>
<td>C1</td>
<td>83.8</td>
<td>98.3</td>
</tr>
<tr>
<td>C2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C3</td>
<td>10.8</td>
<td>17.4</td>
</tr>
</tbody>
</table>

* After incubation of equal volumes of either *A. laidlawii* cells (0.4 mg/ml), *A. laidlawii* membrane lipids (0.4 mg/ml), or *A. laidlawii* membrane protein (0.5 mg/ml) and normal GPS for 30 min at 37°C, C1, C2, and C3 were determined and compared to buffer controls.
in normal GPS. It had been previously shown that fresh GPS exhibited mycoplasmacidal activity for *M. pneumoniae* as well (8). To exclude the possibility that the cidal effect of GPS on *A. laidlawii* was due to antibodies with low specificity mostly directed against *M. pneumoniae*, the mycoplasmacidal effects of GPS and GPS absorbed with *A. laidlawii* were tested. A significant decrease in viability of *M. pneumoniae* was observed with both fresh GPS and GPS absorbed with *A. laidlawii* (Table 4). The data indicate that the specific factors in GPS involved in the killing of *M. pneumoniae* or *A. laidlawii* are different.

**DISCUSSION**

Fresh GPS has most frequently been used as the source of complement to enhance the effect of antibodies on mycoplasmas (1, 6, 8, 12, 15, 20, 34). However, fresh GPS alone exhibits toxic activities directed against some mycoplasma species (6, 11, 15).

Several studies on the mechanism of this effect have previously been performed on *M. pneumoniae*. The activity of GPS against *M. pneumoniae* could not be absorbed with whole organisms. This suggested that antibodies in normal GPS do not play a great role in this reaction. It could also be shown that lysolectin was not responsible for the killing effect of fresh GPS on *M. pneumoniae*.

Recently, Bredt and Bitter-Suermann (4) demonstrated that *M. pneumoniae* is able to activate the alternative pathway of the complement sequence. In addition, they suggested that efficient killing of *M. pneumoniae* depends on an intact classical pathway, triggered either by minimal amounts of antibodies or by another unknown mechanism in normal GPS. Rounding, however, is produced by the alternative pathway (C3 to C9) of the complement sequence or, possibly, by unknown processes on the cell surface, triggered by an incomplete classical pathway (C1 to C4). In both cases, C3 and, possibly, C5 play a role in the reaction.

### Table 3. Determination of antibodies to *A. laidlawii* by immune adherence, using normal GPS from animals of different ages

<table>
<thead>
<tr>
<th>GPS source (age)</th>
<th>Immune adherence titer (reciprocal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>32</td>
</tr>
<tr>
<td>5 weeks</td>
<td>16</td>
</tr>
<tr>
<td>5 weeks</td>
<td>32</td>
</tr>
<tr>
<td>5 weeks</td>
<td>64</td>
</tr>
<tr>
<td>4 months</td>
<td>32</td>
</tr>
<tr>
<td>5 months</td>
<td>256</td>
</tr>
<tr>
<td>5 months</td>
<td>1,024</td>
</tr>
</tbody>
</table>

### Table 4. Effect of fresh GPS and GPS preincubated with *A. laidlawii* on *M. pneumoniae*

<table>
<thead>
<tr>
<th>Complement prepn</th>
<th>Dilution</th>
<th>Decrease in <em>M. pneumoniae</em> viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh GPS</td>
<td>1:2</td>
<td>&gt;99.9</td>
</tr>
<tr>
<td>Fresh GPS</td>
<td>1:20</td>
<td>98.3</td>
</tr>
<tr>
<td><em>A. laidlawii</em></td>
<td>1:20</td>
<td>95.4</td>
</tr>
</tbody>
</table>

Our results obtained on *A. laidlawii* demonstrate that the classical pathway of the complement sequence is activated by antibodies in fresh normal GPS. These antibodies belong to the IgG class of immunoglobulins and are directed against the membrane lipids of the organisms, especially against the phospholipid fraction. These results are consistent with previous data on the surface structure of *A. laidlawii*. The organisms were shown to bind large quantities of the basic proteins lysozyme and cytochrome c, mainly to the polar head groups of their membrane phospholipids (28).

Using polycationic ferritin or positively charged ferric oxide hydroxols in propionic acid as probes, we have demonstrated by electron microscopy a dense and rather homogeneous layer of anionic binding sites on the membrane surface of *A. laidlawii*. With chemical and enzymatic techniques, the anionic sites were identified to be mainly lipid phosphate groups of, e.g., phosphatidylglycerol (32, 33).

Antibodies induced by and directed against the isolated phospholipids of *A. laidlawii* reacted with whole *A. laidlawii* cells, as demonstrated by the metabolism inhibition and the mycoplasmacidal tests (11). According to our previous studies with lectins (31) and specific antiglycolipid antibodies (1), the glycolipids of *A. laidlawii* do not significantly contribute to the surface architecture of this organism.

Activation of the alternative pathway of the complement sequence does not play a great role in the killing effect of fresh GPS on *A. laidlawii*. This could be demonstrated by the use of zymosan and by determination of the reduction of C1, C4, C2, and C3 in normal GPS and of C1, C2, and C3 in C4-deficient GPS in the presence of *A. laidlawii*.

Antibodies to *A. laidlawii* are already detectable in 1-week-old guinea pigs. These antibodies are presumably of maternal origin. The titers increase with age.

During previous studies on experimental infection of guinea pigs with *M. pneumoniae* we did not isolate *A. laidlawii* from the noses, lungs, kidneys, livers, or brains of normal guinea pigs. There is only one short communication on the isolation of *A. laidlawii* from the vaginae of...
guinea pigs (19). The antibodies to *A. laidlawii* in normal guinea pigs may be due to natural infection with these organisms. The antibodies in normal GPS may have also been induced by antigenic determinants of other microorganisms or food antigens (10, 16, 29). They may be due to immunological cross-reactions, which were observed between various phospholipids, e.g., phosphatidylglycerol, phosphatidylglycerolphosphate (cardiolipin), and the widespread phosphoglycolipid, glycerylphosphoryldiglycerol, as well as further substances containing the highly immunogenic phosphate groups, i.e., teichoic acids and deoxyribonucleic acid (17, 18, 30, 35; H.-G. Schiefer, U. Gerhardt, and H. Brunner, submitted for publication).

Substantial support for this assumption is given by experiments on *M. pneumoniae*, the glycolipids of which have antigenic determinants in common with *Staphylococcus aureus*, *Streptococcus pyogenes*, parsnips, and carrots (16).

It can be speculated that the antibodies to *A. laidlawii* in normal GPS provide protection, since the organisms are killed by these antibodies in the presence of complement.

**LITERATURE CITED**


