Immunological Analysis of Plasma Membranes of a T-Strain of Mycoplasma (Ureaplasma urealyticum)

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The cell membranes of a T-strain of mycoplasma, obtained by ultrasonic disruption, were as effective as whole organisms in eliciting metabolism-inhibiting and complement-fixing antibodies. The soluble fraction separated from cell membranes by centrifugation at 35,000 × g showed a minor ability to elicit an antibody response as measured by metabolism inhibition and complement fixation tests. After a further centrifugation at 100,000 × g, the immunogenic activity of the soluble fraction was completely lost. Immunogenic determinants in mycoplasma membranes could also be demonstrated by adsorption tests: cell membranes were more effective than soluble fractions in adsorbing antibody capacity from the immune sera against whole cells. It has been shown by further experiments that cell membranes have at least two major antigenic determinants, which differ either in chemical nature or in capacity to adsorb and evoke antibodies, characterized by different serological behaviors.

Membranes of several mycoplasma species have been isolated by different procedures and examined for their antigenic properties (8).

In spite of the difficulties in separating the cell membrane from the cytoplasm, localization studies of the antigenic activities have shown that immunogens eliciting the production of metabolism-inhibiting (MI) and growth-inhibiting as well as complement-fixing (CF) antibodies (8) are located in the cell membrane. Chemical analysis of mycoplasma membranes shows that, like other biological membranes, they are essentially built of protein and lipid (7).

A previous report demonstrated that the CF activity of a T-strain of mycoplasma was associated with its own lipid component (10), and since virtually all the lipid content is located in the membrane (7), it is likely that this activity is membrane bound.

This possibility prompted a study of immunological characterization of the T-strain mycoplasma membrane.

The results of our investigations are presented in this paper.

MATERIALS AND METHODS

Organisms and growth conditions. The T-strain of mycoplasma studied was P108, isolated from human vagina in our laboratory. This strain can be classified as Ureaplasma urealyticum on the basis of its habitat, cultural and biochemical properties, and serological cross-reactivity with serotype VI (strain Pi, ATCC 27818) of U. urealyticum.

The organisms, grown in Trypticase soy medium (11) containing 5% (vol/vol) horse serum, were harvested after 16 to 20 h of incubation at 37°C, washed three times in 0.25 M NaCl in the cold, and suspended in a small volume of the same solution.

Isolation of cell membranes and soluble cell proteins. Soluble proteins and cell membranes of strain P108 were obtained by ultrasonic disruption of the organisms. An organism suspension in 0.25 M NaCl was treated in a Biosonik II ultrasonic disintegrator (18.6 kHz/s) at 4°C for 15 1-min periods, with 1-min intervals to avoid heating. The soluble fraction was separated from the cell membranes by centrifugation at 35,000 × g for 2 h at 4°C. Part of the soluble fraction was subjected to an additional centrifugation at 100,000 × g for 2 h at 4°C. All the soluble fractions were freeze-dried and stored at −20°C.

The cell membranes were washed three times in 0.05 M NaCl in 0.01 M phosphate buffer, suspended in the same buffer to a concentration of 3 to 4 mg of membrane protein per ml, and stored at −20°C until used.

Analytical methods. Protein was determined by the Polin phenol method of Lowry et al. (4) against a standard of crystalline bovine plasma. Membrane lipids were extracted with chloroform-methanol (2:1) (11).

Defatted membrane proteins. The techniques of Fleischer et al. (2), Rega et al. (9), and extraction of lipids with chloroform-methanol (2:1) were employed to free membrane proteins from lipids.

Preparation of antigens. P108 organisms washed three times in 0.25 M NaCl and suspended in the same solution were used as whole-cell antigen. The preparations of antigens containing cell membranes, soluble cell proteins, and membrane proteins free from lipids were as described above. Antigens for immunization were prepared by dilution of the above preparations to 1 mg of protein per ml of
phosphate-buffered saline, pH 7.4. To prepare lipid antigen, lipid amounts equivalent to 500 μg were pipetted into 25-ml flasks. The chloroform-methanol (2:1) was removed in vacuo by using a rotary evaporator. To the thin, dry lipid film, 1 ml of deionized water containing 1 mg of human serum albumin at 37°C was added. A gentle shaking was followed by sonic treatment for 4 to 5 min in a Biosonik II ultrasonic disintegrator. The immunization schedule was as previously described (10).

Adsorption of antiserum. Antiserum were adsorbed against membranes by addition of 0.1 ml of antiserum to the sedimented antigen (containing 1 mg of protein). Adsorption against soluble cell protein and membrane proteins free from lipid antigens was done by mixing equal volumes of antiserum and protein solution (10 mg/ml). The mixture was shaken at 37°C for 1 h, transferred at 4°C overnight, and then centrifuged at 25,000 x g for 15 min. The supernatant fluid was separated and adsorbed against another portion of antigen, as described above.

For adsorption of antiserum against membrane lipids, dried lipids, obtained from membranes containing 3 mg of protein per ml, were emulsified in 0.3 ml of antiserum, and adsorption was carried out as described above. The adsorbed sera were kept at -20°C until used.

Serological tests. The CF test was carried out in a microtitration system, using the whole-cell suspension as antigen (10).

The MI test was carried out as described by Purcell et al. (6).

RESULTS

Immunogenicity of cell membranes and soluble protein fractions. The antiserum obtained by immunization of rabbits with P108 organisms, cell membranes, and soluble protein fractions were tested for CF and MI with P108 organisms. Heterologous controls were also used, i.e., Mycoplasma hominis (strain PG21), grown in the medium with 1 g of L-arginine per 100 ml, and horse serum, to assay the reactivity of the T-strain antigen with horse serum or medium components.

Table 1 shows that cell membranes of strain P108 were as effective as whole cells in eliciting MI and CF antibodies. The soluble fraction showed a minor ability to elicit an antibody response, as measured by the above-mentioned tests. Furthermore, the soluble fraction subjected to a further centrifugation at 100,000 x g failed to elicit any significant antibody response, suggesting that the supernatant fractions were contaminated by minute membrane particles derived from fragmentation by ultrasonic disruption.

However, the rabbit antiserum were also slightly cross-reactive in the CF test with heterologous controls. The reactivity was completely abolished when the immune sera were previously adsorbed with horse serum; therefore, for the subsequent experiments only immune sera previously adsorbed with horse serum were used.

Localization of immunogenic determinants in mycoplasma membranes could also be demonstrated by adsorption tests. CF and MI antibodies of serum against whole cells were completely adsorbed by washed membranes (Table 2). The activity of the soluble fractions (1 and 2) in adsorbing the antibodies of the antiserum to whole cells could be detected only with the CF test, since the MI test could not be performed.

<table>
<thead>
<tr>
<th>Table 1. Serological response of rabbits to cell fractions of P108 mycoplasma and control preparations as measured by CF and MI tests</th>
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<tbody>
<tr>
<td><strong>Antigen</strong></td>
</tr>
<tr>
<td>****</td>
</tr>
<tr>
<td>P108</td>
</tr>
<tr>
<td><em>M. hominis</em></td>
</tr>
<tr>
<td>Horse serum</td>
</tr>
</tbody>
</table>

* Fraction separated after centrifugation at 35,000 x g for 30 min.
* b Fraction separated after centrifugation at 100,000 x g for 2 h.
* c CF antigen was a whole-cell suspension.
due to the urease activity present in the adsorbed serum, which caused a rapid pH shift of the medium to alkalinity.

Table 2 shows again that only soluble fraction 1 was able to adsorb some CF antibodies, thus confirming the hypothesis that this soluble fraction was contaminated with minute membrane fragments.

Adsorption of antibody by membrane fractions. To establish the nature of the immunogens, lipids extracted from membranes and several defatted membrane protein preparations were examined for MI and CF antibody-adsorbing capacity. Lipids extracted from membranes reduced the CF antibody titer but did not affect the MI antibody titer (Table 3). The lipid-free membrane preparations obtained after chloroform-methanol or 10% water in acetone extraction showed a low capacity to adsorb either MI or CF antibody, whereas defatted membrane protein preparations with cold n-butanol alcohol did so most effectively (Table 3).

Immunogenicity of membrane fractions. Since lipids extracted with chloroform-methanol from P108 membranes could adsorb only the CF antibodies, and defatted membrane preparations with cold n-butanol could adsorb both MI and CF antibodies, our subsequent experiments were planned to determine whether an antibody response could be elicited by the injection of lipids and defatted membrane proteins into rabbits. However, as the serologically active lipids can be regarded as haptens, we tried to evoke an immune response to lipids in rabbits by the injection of a mixture consisting of lipids extracted with chloroform-methanol (2:1) with human serum albumin.

Lipid antigen and defatted membranes obtained by n-butanol extraction retained most of the ability of whole membranes to elicit antibodies in rabbits (Table 4). Moreover, only CF antibodies could be detected after the injection of lipid-protein complex, whereas defatted membrane proteins elicited both MI and CF antibodies.

**DISCUSSION**

Our results show that animals immunized with membranes produce antibodies reacting with P108 organisms in CF and MI tests.

A comparison of the titers induced by soluble and membrane fractions shows that immunizing antigens are located in the membrane, just as the major immunogens of other organisms tend to be located on the surface. That antigenic determinants are located in the mycoplasma membrane can also be demonstrated by adsorption tests. Thus, the antibodies from antisera to whole cells were most effectively adsorbed by membranes, but hardly at all by the soluble fraction. It should be noticed, however, that the urease activity still present in the anti-P108 organism antisera after adsorption with the soluble fraction did not allow the performance of the MI test.

The adsorption tests performed with different membrane preparations have also shown that the cell membrane is the site of at least two major antigenic determinants: one, extracted by chloroform-methanol, could eliminate only the CF antibodies, and the second one, obtained in the defatted membrane, could adsorb both MI and CF antibodies. However, the defatted protein fractions varied largely in their capacity to adsorb MI and CF antibodies. There is no doubt that solubilizing membrane proteins without causing denaturation or conformational changes is a very difficult problem. Extraction of the lipids with cold n-butanol was gentle enough for the membrane proteins to retain their MI and CF adsorbing capacity. Moreover, this defatted fraction retained a significant part of its immunogenic ability.

The current intensive study of biological membranes will probably sweep away the tech-

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**Table 3. Adsorption of MI and CF antibodies by membrane fractions of P108 mycoplasma**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Reciprocal of antibody titer</th>
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<tr>
<td></td>
<td>CF</td>
</tr>
<tr>
<td>Nonadsorbed</td>
<td>160</td>
</tr>
<tr>
<td>Adsorbed with:</td>
<td></td>
</tr>
<tr>
<td>10% water-acetone-extracted</td>
<td>64</td>
</tr>
<tr>
<td>membranes</td>
<td></td>
</tr>
<tr>
<td>n-Butanol-extracted membranes</td>
<td>16</td>
</tr>
<tr>
<td>(aqueous phase)</td>
<td></td>
</tr>
<tr>
<td>Chloroform-methanol-extracted</td>
<td>64</td>
</tr>
<tr>
<td>membranes</td>
<td></td>
</tr>
<tr>
<td>Membrane lipids (chloroform-</td>
<td>16</td>
</tr>
<tr>
<td>methanol-extracted)</td>
<td></td>
</tr>
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**Table 4. MI and CF antibody titer to various membrane fractions**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>No. of animals</th>
<th>Antibody titer (mean and range of reciprocal)</th>
</tr>
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<tbody>
<tr>
<td>Lipid antigen</td>
<td>3</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Lipid alone</td>
<td>2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Membrane (n-butanol-extracted)</td>
<td>3</td>
<td>288 (128-512)</td>
</tr>
<tr>
<td>Cell membranes</td>
<td>2</td>
<td>1,536 (1,024-2,048)</td>
</tr>
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**Note:** Numbers in parentheses indicate changes of 160 units.
nical difficulties that now impede the characterization of membrane proteins in general and the serologically active one in particular.

Some of the new methods based on the use of proteolytic enzymes and labeling agents combined with electrophoretic analysis of membrane polypeptides in polyacrylamide gels, recently used to study protein disposition in the membrane of *Acholeplasma laidlawii* (1), may be used to determine the exact position of the protein antigen in or on the membrane.

Our results demonstrate, too, that the lipids are able to adsorb and evoke CF antibodies. Such results are in agreement with our previous report (10), which showed the lipids' ability to react in the CF test with an antiserum against the whole organism.

However, since they cannot adsorb MI antibodies or elicit an MI response, their disposition on the membrane surface seems to be different from that of the lipids of *Mycoplasma pneumoniae*, which can adsorb and evoke both MI and CF antibodies.

Although immunological data are available for only some of the known mycoplasmas, the generalization seems warranted that in most species proteins are the major antigens responsible for eliciting the production of MI and CF antibodies (8). Lipids play a minor role, if any, in the antigenic activity of the mycoplasmas tested so far. The exceptions are *M. pneumoniae* and *Mycoplasma fermentans* (3), in which the major antigen was found in the lipid fraction.

In our *Ureaplasma* strain, it seems that both chemical compounds, lipids and proteins, are involved in a different way in antibody formation.

Such information indicates that the organisms in the order *Mycoplasmatales* are surprisingly heterogeneous, and the chemistry of the major determinants may eventually provide further criteria for classification.

**LITERATURE CITED**


