INTRACELLULAR M PROTEIN OF GROUP A STREPTOCOCCUS

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ABSTRACT

Fox, Eugene N. (University of Chicago, Chicago, Ill.). Intracellular M protein of group A Streptococcus. J. Bacteriol. 85:536-540. 1963.—A heat-labile M protein antigen in protoplasts of a type 14 strain of group A Streptococcus has been demonstrated in a soluble form in the cytoplasm, and also bound to the protoplasmic membrane. When trypsinized whole cells (from which the M protein on the cell wall had been digested) or protoplasts were extracted with hot HCl, no M protein antigen was detected, although the antigen was routinely extracted from the cell walls of normal streptococci by the hot acid procedure. Various serological techniques, including the use of fluorescein-labeled type-specific antisera, were employed to demonstrate the M protein antigen in association with the membrane from osmotically lysed protoplasts.

Current work in this laboratory has been concerned with the synthesis of proteins, such as the M antigen, by subcellular fractions of hemolytic streptococci; concomitant with this endeavor has been the identification and localization of the M protein antigen within the streptococcal cell. The M protein is most commonly identified as a cell-wall constituent (see, for example, Lancefield, 1954), although its intracellular existence and origin have been obscure. Certain strains of streptococci have been found to elaborate the M protein into the growth medium (Olarte, 1948), but whether this phenomenon represents a dissolution of excess antigen from the cell, cell autolysis, or excretion has never been defined. Friemer, Krause, and McCarty (1959) adequately demonstrated that L forms of group A streptococci growing in agar excrete the M antigen into the surrounding medium, apparently owing to the absence of a cell wall for the deposition of the antigen.

If group A streptococci are first incubated in a trypsin solution, no M antigen is detected when the cells are extracted with hot dilute HCl according to routine serological typing procedures (Lancefield, 1943; Fox and Krampitz, 1956). This fact would indicate a peripheral location for the M protein, but the previously cited evidence seems to imply an intracellular synthesis. The data to be presented may clarify these facts: soluble intracellular M protein and M protein bound to the protoplasmic membrane have been found; these antigens so demonstrated are relatively labile, if extracted by the hot HCl procedure.

MATERIALS AND METHODS

Streptococci and antisera. Types 14 and 24 group A streptococci virulent for mice were maintained in storage in Todd-Hewitt Broth at -70 C. Antisera were prepared in rabbits according to a modified procedure of Swift, Wilson, and Lancefield (1943). The initial dose was administered with Freund's adjuvant (complete) in the hind foot pad; a booster dose 3 weeks later was given intravenously without adjuvant. Usually two of these series were sufficient to produce high-titer antisera.

Cultivation and trypsinization of cells. These procedures are outlined in a previous communication (Fox, 1961).

Solutions. Three solutions were used: (i) buffered saline: 0.85% NaCl in 0.01 M Na2PO4, (pH 7.0); (ii) buffered sucrose: 1 M sucrose in buffered saline; (iii) tris(hydroxymethyl)aminomethane (tris) buffer: 0.1 M tris buffer (pH 7.9) containing 10^{-4} M MgCl2. Protein concentrations were measured according to the method of Lowry et al. (1951).

Preparation of protoplasts. The phage-associated lysin described by Goorder and Maxted (1958) and Krause (1958) was used to obtain protoplasts of group A streptococci. A modification of the technique of Markovitz and Dorfman (1962) was used to obtain lytic enzyme...
of exceptionally high potency. Two liters of \(2 \times 10^9\) group C streptococci (the phage and group C *Streptococcus* were a gift from R. M. Krause, Rockefeller Institute) per ml in the exponential phase of growth were infected with the group C phage at a multiplicity of 3:1 (phage per cell). For this purpose, phage preparations with titers up to \(10^{12}\) particles per ml could be obtained in the absence of lytic enzyme by harvesting the phage from confluent plaques on soft agar layer plates (Adams, 1959). At the end of 25 min (i.e., before the onset of lysis) the infected culture was chilled to 5 C by pouring it over ice, and was then immediately centrifuged.

The packed cells were osmotically fragile and lysed when resuspended in 30 ml of buffered saline containing 5 \(\mu\)g per ml of deoxyribonuclease, \(10^{-3}\) M MgCl\(_2\), and \(10^{-3}\) M 2-mercaptoethanol. The suspension was allowed to stand at room temperature for 15 min and centrifuged at 10,000 \(\times\) g for 30 min. The clear supernatant containing the enzyme was distributed in 1-ml fractions and stored at \(-20\) C. This concentrated lytic enzyme, when diluted (1:1,000) in buffered saline containing \(10^{-3}\) M 2-mercaptoethanol, decreased the optical density of a suspension (5 \(\times\) \(10^9\) streptococci per ml) by 50\% in 5 min.

For the preparation of protoplasts, 4 g of packed cells of group A streptococci in 100 ml of sucrose buffer were treated with 1 ml of concentrated enzyme for 60 min at 37 C, and then washed twice in 50-ml volumes of sucrose buffer.

**Fraction of protoplasts.** The various cell fractions were prepared from the osmotically fragile protoplasts, according to the flow diagram in Fig. 1.

**Results**

**Intracellular M protein.** The protoplast lysate from which the microsomes had been removed was adjusted to pH 5.0 with 1 M acetic acid, and the precipitate was dissolved in saline at a concentration of 2.8 mg per ml. The solution was divided into three portions. One sample (A) was the untreated control; to the second (B) were added 5 \(\mu\)g per ml of ribonuclease; no enzyme was added to the third portion (C). The fractions were incubated at 37 C for 30 min. To fractions B and C was added 1 \(\times\) HCl to pH 2.5. The solutions B and C were heated at 95 C for 10 min, cooled, and neutralized with 0.1 M NaOH. Insoluble material was removed by centrifugation; a considerably larger amount of denatured material was removed from fraction C. The protein concentration of the three solutions was adjusted to 1 mg per ml, and they were placed in reaction with antisera in a capillary precipitin test (Table 1). The heat lability of the M antigen in fractions B and C is obvious, and the greater diminution of antigen in C may be

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**Washed protoplasts from 4 g (wet wt) of streptococci were lysed in 25 ml of buffered saline containing 50 \(\mu\)g of deoxyribonuclease and \(10^{-3}\) M MgCl\(_2\); room temp 15 min**

- **Centrifuged for 30 min at 10,000 \(\times\) g, 0 C**
  - Pellet (membranes) washed twice in buffered saline, 0 C
  - Supernatant centrifuged at 105,000 \(\times\) g for 70 min, 0 C
- Pellet (microsomes) washed in tris buffer, centrifuged at 105,000 \(\times\) g, 0 C
  - Supernatant dialyzed for 12 hr in buffered saline, 5 C
  - 70\% saturated \((NH_4)_2SO_4\) for 60 min, 0 C, centrifuged
  - Precipitate (containing M antigen) dissolved in saline
- **Supernatant discarded**

**FIG. 1. Flow diagram for cell fractionation.**
TABLE 1. Precipitin reactions of intracellular M protein, type 14

<table>
<thead>
<tr>
<th>M protein fraction</th>
<th>Antiserum*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type 14 (adsorbed)</td>
</tr>
<tr>
<td>A</td>
<td>+++</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>±</td>
</tr>
</tbody>
</table>

* Results expressed as estimated degree of precipitin reaction in capillary tubes.

In view of the fact that the type-specific serum was absorbed with heterologous type 24 whole cells, antibodies to various nonspecific intracellular antigens would not necessarily have been removed by the absorption procedure. Therefore, agar-diffusion plates were prepared to identify the lysate antigen(s) which precipitated with type-specific serum. Figure 2 is a replication of an agar-diffusion plate in which various preparations of M protein were compared with the intracellular soluble M antigen. A highly purified preparation of type 14 M protein (Fox, 1961) gave a single sharply defined precipitin line with type 14 antiserum. This precipitin band was contiguous with the M protein fractions, including the intracellular protein from the lysed protoplasts. However, hot acid extracts of trypsinized cells (from which the protoplasts were prepared) gave no line of identity with the M protein, although several antigens including the group A carbohydrate were demonstrable. These results demonstrate several interesting properties involved in the association of M antigen and the bacterial cell; namely, that the soluble intracellular M protein is not observed in the hot acid extracts of trypsinized cells, although the M antigen is present in easily demonstrable quantities in the soluble protoplasm, but appears to be relatively labile in hot acid.

M protein on membranes. Streptococcal protoplasmic membranes were prepared from trypsinized streptococci in the manner previously described. After several washings in large volumes of saline, the membranes were suspended in saline at a concentration such that a dilution of

TABLE 2. Adsorption of fluorescein-labeled antibody to protoplast membranes*

<table>
<thead>
<tr>
<th>Membrane (serological type)</th>
<th>Pretreatment</th>
<th>Fluorescent globulin</th>
<th>Intensity of fluorescence†</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Saline</td>
<td>Anti-14</td>
<td>10.4</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>14 Antiserum</td>
<td>Anti-14</td>
<td>7.2</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>14 Antiserum</td>
<td>Normal</td>
<td>2.4</td>
</tr>
<tr>
<td>24</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Saline</td>
<td>Normal</td>
<td>2.3</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Portions (1 ml) of a membrane suspension (5.2 mg of protein) were incubated at 37°C for 15 min with 0.2 ml of solution (pretreatment column), and then 0.2 ml of fluorescein-labeled antitype 14 globulin or labeled normal rabbit globulin was added. After 15 min of incubation, the suspensions were diluted with 6 ml of cold saline, centrifuged, and washed twice with saline at 0°C. The fluorescent fraction was eluted with 0.1 M NaOH, buffered at pH 8.6 with borate, and read in an Amino-Bowman Spectrophotofluorometer (Fox, 1962).

† Expressed as per cent transmission.
1:10 gave a Klett density value of approximately 100 units ("66" filter), which corresponded to a concentration of 3.2 mg of protein per ml. These membranes, when extracted with hot HCl, yielded only traces of precipitate in a capillary precipitin test. A more conclusive technique for observing the apparently labile M protein associated with the membranes was a fluorescent antibody adsorption procedure developed in this laboratory (Fox, 1962). The γ-2 globulin fraction of type-specific antibody was labeled with fluorescein, adsorbed specifically to the membranes, and then eluted and measured in a fluorometer. Various controls were carried out to demonstrate the type-specificity of the reaction and to establish the degree of nonspecific fluorescein adsorption. These controls included the use of fluorescein-tagged normal globulin, and preincubation of membranes with untagged specific antiserum, the latter to compete with the subsequent adsorption of the labeled antibody on the membranes. These data are summarized in Table 2. The figures for per cent transmission are the average of three samples from each reaction mixture, and each set agreed well within the statistical error. The Aminco-Bowman Spectrophotofluorometer used for these measurements, with a photomultiplier attachment, was accurate from 0.001 to 100% transmission.

The data demonstrate a type-specific reaction of antibody with protoplast membranes; the adsorption could be partially inhibited by a competitive reaction with unlabeled antibody, although this antibody had no effect on the nonspecific absorption of the fluorescein-tagged normal globulin. Membranes from a heterologous type 24 strain adsorbed a limited amount of fluorescent protein which was not altered by the preliminary addition of type 14 antiserum, and that amount of fluorescence did not exceed the value obtained with labeled normal globulin and type 24 membranes.

Antigenic reactivity of the microsomes. The microsomes were washed in tris buffer and collected by high-speed centrifugation as outlined in Fig. 1. In the absence of magnesium, the particles were unstable. This fact, plus the method of obtaining the microsomes, was our basis for defining these particles as such. To 0.5-ml volumes of serially diluted antisera was added 0.1 ml of the microsome suspension containing 1.2 mg of protein per ml; the tubes were incubated at 37 C for 2 hr, and overnight at 5 C. The next day, the tubes were centrifuged at 1,500 × g for 10 min, and the flocculation pattern on the bottom of the tubes was read. The results (Table 3) demonstrate that only a small fraction of the microsomes are precipitated by type-specific serum. A more intense reaction with heterologous unadsorbed serum occurred as predicted: this serum contains antibodies to a large number of nonspecific streptococcal proteins, all of which are most likely associated with the microsomes.

**TABLE 3. Flocculation reaction of type 14 streptococcal microsomes with antistreptococcal sera**

<table>
<thead>
<tr>
<th>Dilution of antiserum</th>
<th>Antiserum*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type 14 (adsorbed)</td>
</tr>
<tr>
<td>1:5</td>
<td>++</td>
</tr>
<tr>
<td>1:10</td>
<td>+</td>
</tr>
<tr>
<td>1:20</td>
<td>±</td>
</tr>
<tr>
<td>1:40</td>
<td>–</td>
</tr>
<tr>
<td>1:80</td>
<td>–</td>
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<tr>
<td>1:160</td>
<td>–</td>
</tr>
<tr>
<td>1:320</td>
<td>–</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
</tr>
</tbody>
</table>

* Results expressed as estimated intensity of the flocculation reaction.

**Discussion**

Simple fractionation and serological procedures have been used to demonstrate a heat- and acid-labile M antigen found intracellularly and in association with the streptococcal protoplasmic membrane. Inability to detect the M antigen in hot acid extracts of trypsinized cells may also be due to the adsorption of the antigen on denatured protoplasm. (Myoda and Krampitz, personal communication; these authors report that extracts of trypsinized streptococci disrupted by various mechanical methods react with type-specific sera. The extracts lose this activity when treated with hot acid and neutralized after centrifugation.)

The expedience of trypsinizing whole cells was undertaken before the protoplasts were prepared, to insure an intracellular preparation free of contaminating M protein from the cell wall. Plate counts of viable cells in a protoplast preparation indicated that a very small fraction...
of cells (less than 10⁶ in a suspension of 10⁶) are refractory to the phage-associated lysozym. The M protein on the protoplasmic membranes was therefore not of cell-wall origin. Freimer et al. (1959) were unable to demonstrate any M protein associated with the membranes. They used the standard method of hot acid extraction, and their negative results may well have been due to the lability of the intracellular M protein.

The data presented in this communication do not allow one to identify an intracellular site of synthesis of the M antigen. However, Markovitz and Dorfman (1962) supplied evidence attesting to the metabolic activity of isolated streptococcal membranes through their demonstration of hyaluronic acid synthesis from nucleotide precursors. In current experiments, I have observed that, with isotopically labeled amino acids added to cell-free streptococcal fractions, the only fraction to take up the label significantly is the protoplasmic membrane. The system is dependent on an adenosine triphosphate-generating system, the "pH 5 enzymes," and amino acids. The microsomes from the lysate apparently do not participate; the system is inhibited by ribonuclease and chloramphenicol. As yet I have been unable to effect a release of labeled protein from the membranes, and it remains to be seen whether these structures are the site of M protein synthesis.

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LITERATURE CITED


