IMMUNITY TO THE GROUP B STREPTOCOCCI:
INTERACTION OF SERUM AND MACROPHAGES
WITH TYPES Ia, Ib, AND Ic*

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The emergence of the group B streptococci as significant and prevalent agents in human perinatal and neonatal infections is relatively recent (1) and has stimulated renewed interest in the biology of these organisms. However, their antigenic composition has been studied for several decades. Shortly after characterizing this serological group of streptococci (2), Lancefield identified three distinctive serotypes, I, II, and III, on the basis of specific carbohydrate (CHO)1 antigens (3) and subsequently separated type I into Ia and Ib (4). Recently, Wilkinson and her associates have identified a third member of the type I complex, type Ic, which is related to type Ia by the mutual possession of IaCHO and to type Ib by the presence in both of Ibc protein (5, 6). All three type I serotypes share IabcCHO (7). Other cellular antigens of the group B streptococci have been defined, (8) and some have been isolated and analyzed by immunological techniques (9-13).

This paper summarizes a series of experiments that were designed to develop a quantitative assay of phagocytosis and to identify the critical microbial antigens and serum requirements for the opsonization of group B streptococci. This report deals with streptococci of types Ia, Ib, and Ic and, to facilitate the presentation and discussion of data, Table I summarizes the principal cellular antigens of these serotypes, as defined in the laboratories of Lancefield et al. (7) and of Wilkinson and Eagon (6).

Materials and Methods

Streptococcal Strains. Prototype group B streptococcal strains were provided by Dr. R. C. Lancefield: 090, type Ia; H36B, type Ib; A909, type Ic; 18RS21, type II; D136C, type III. Working stocks were maintained on sheep blood agar at 4°C and subcultured at monthly intervals.

Animals. Adult, female, New Zealand albino rabbits (8-9 pounds) were used as a source of normal rabbit serum (NRS) and antiserum. Smaller (6-7 pounds), unsexed animals were the source of alveolar macrophages.

Preparation of Antisera. Rabbits were immunized intravenously with formalin-killed streptococci by the method of Lancefield (3). The injection schedule was 0.5 ml of bacterial suspension on 3 successive days followed at weekly intervals by 1.0 ml on 3 successive days. Immunizations were continued for 1 or 2 mo or until antiserum demonstrated strong homologous reactions when set up

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1 Abbreviations used in this paper: CFU, colony-forming units; CHO, carbohydrate; FCS, fetal calf serum; NRS, normal rabbit serum.
**TABLE I**

**Distribution of Cellular Antigens of Group B Streptococcal Types Ia, Ib, and Ic**

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Type-specific carbohydrate</th>
<th>Common carbohydrate</th>
<th>Common protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>IaCHO</td>
<td>IsbCHO</td>
<td>-</td>
</tr>
<tr>
<td>Ib</td>
<td>IbCHO</td>
<td>IsbCHO</td>
<td>Ibc protein</td>
</tr>
<tr>
<td>Ic</td>
<td>IaCHO</td>
<td>IsbCHO</td>
<td>Ibc protein</td>
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* Based upon the reports of Lancefield et al. (7) and Wilkinson et al. (6).

with hot-acid extracts of all five prototype strains in an Ouchterlony double-diffusion test. Sera were absorbed with heat-killed streptococci in a proportion of 1 vol serum:1 vol packed bacteria; these were mixed and left at room temperature for 18 h (14). Sera were studied unheated and after heating for 30 min in a 56°C water bath.

**Preparation of Radiolabeled Streptococci.** 2 µCi/ml of uniformly labeled L-[14C]leucine (New England Nuclear, Boston, Mass.) was added to 5 ml of RPMI medium no. 1640 without leucine (Grand Island Biological Co., Grand Island, N. Y.). This was inoculated with 0.1 ml of an 18-h streptococcal broth culture (Todd-Hewitt, Difco Laboratories, Detroit, Mich.), incubated overnight at 37°C, and harvested by centrifugation at 1,500 g for 10 min. The sedimented bacteria were washed three times with saline and suspended in the original volume of saline. After dilutions and pour plates were made to determine the number of colony-forming units (CFU), the streptococci were killed by heating at 60°C for 1 h. Bacterial purity and killing were confirmed by culture. Suspensions of labeled streptococci were stored at 4°C, and the integrity of the labeled bacteria was checked at least weekly by centrifugation at 1,500 g for 10 min; this consistently precipitated >95% of counts per minute.

**Bacterial Phagocytosis by Cultured Alveolar Macrophages.** The technique of Reynolds and Thompson (15) was modified by the use of glass coverslips rather than plastic dishes as the supporting substrate for macrophages. This change obviated the removal of macrophages from substrate before scintillation counting and was considered valid if the majority of macrophages could be demonstrated to remain adherent and viable during incubation with serum and bacteria and subsequent washing.

Rabbits were anesthetized with 60 mg of sodium pentobarbital intravenously and killed by air embolism. Under aseptic conditions, the trachea was promptly isolated, cannulated with a pediatric endotracheal tube (Shiley Laboratories, Inc., Santa Ana, Calif.), and lavaged with four 50-ml vol of saline. The alveolar macrophages were separated from this suspension by centrifugation at 550 g for 10 min and washed twice with 10 ml of modified McCoy’s 5A medium containing 10% fetal calf serum (FCS) (both purchased from Grand Island Biological Co.). The FCS had been heated at 56°C for 30 min and filtered through a Seitz asbestos filter (Scientific Products, Irvine, Calif.). Gentamicin sulfate (5 µg/ml) was added to all McCoy’s medium to inhibit the growth of *Bordetella bronchiseptica*, a contaminant of the lung washings in approximately 50% of these experiments.

Short-range macrophage cultures were established on 10.5 × 22-mm glass coverslips (Wheaton Scientific, Div. Wheaton Industries, Millville, N. J.) in 13 × 125-mm siliconized Leighton tubes. Coverslips were precleaned and coated with poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.) to enhance cell adherence (16). Macrophages were counted in a hemocytometer, and approximately 10⁶ in 1 ml of McCoy’s medium with 25% FCS was added to each coverslip. The tubes were loosely stoppered and incubated at 37°C in 5% CO₂ for approximately 18 h, at which time the medium was decanted from all tubes and the coverslips washed three times with McCoy’s medium containing 10% FCS. For each set of macrophage cultures from a single animal, the number of viable, adherent cells per tube was estimated by treating one or two coverslips with 1 ml of 0.25% trypsin in phosphate-buffered saline, pH 7.4, diluting the trypsin-cell suspension with 0.2% trypsin blue in a leukocyte pipette and counting in a hemocytometer. 20-50% (2-5 × 10⁶ per coverslip) of the original macrophage inoculum could be recovered and >99% of the adherent cells were viable as judged by exclusion of dye.
An appropriate dilution of labeled streptococci was mixed with an equal volume of a 1:5 dilution of NRS or antiserum (both in McCoy's medium with 10% FCS) and incubated in a 37°C water bath for 10 min. 0.1 ml of the bacteria-serum mixtures and 0.9 ml of McCoy's medium with 10% FCS were then added to each coverslip for a bacterial CFU:macrophage ratio of 100:1, and the Leighton tubes were agitated gently at 37°C on a rocking platform (Bellco Glass, Inc., Vineland, N. J.). At 0, 30, 60, and 120 min, a number of coverslips were washed three times with McCoy's medium and 10% FCS to remove free bacteria, air dried, and immersed in scintillation vials to determine radioactive uptake by macrophages. At 120 min one or two additional coverslips were trypsinized and the trypsin-cell suspension counted in the presence of trypan blue to rule out excessive loss of macrophages during the experiment. For all experiments reported here, ≥90% of the originally adherent cells survived. Scintillation fluid consisted of equal volumes of toluene (Matheson, Coleman & Bell, Rutherford, N. J.) and Permablend II (Packard Instrument Co. Inc., Downers Grove, Ill.), and counts per minute were determined in duplicate in a Nuclear-Chicago Mark II Liquid Scintillation Counter (G. D. Searle and Co., Des Plaines, Ill.). The extent of each phagocytosis experiment was limited by the quantity of macrophages recovered from a single rabbit and hence the number of coverslip cultures. Therefore, it was not possible to evaluate opsonization of each streptococcal strain by all sera in a single experiment. However, all results reported here were confirmed in additional experiments which are not shown.

Microscopy. At the end of phagocytosis experiments, some coverslips were washed, air dried, treated with Wright's stain, mounted on glass slides, and examined under oil immersion with a Zeiss photomicroscope (Carl Zeiss, Inc., New York). Photomicrographs were made with Kodachrome II professional film (Eastman Kodak Co., Rochester, N. Y.).

Bacterial Killing by Macrophage Suspension. This was assessed by micromodification of the method of Quie et al. (17). An overnight broth culture of unlabeled streptococci and freshly harvested alveolar macrophages were washed and suspended in Hanks' balanced salt solution with 1% gelatin. Similar concentrations of CFU and macrophages (3-4 × 10⁴/ml) and 4% NRS or antiserum in a total vol of 0.5 ml were tumbled in 12 × 75-mm plastic tubes with snap closures (Falcon Plastics, Div. of Bioquest, Oxnard, Calif.) at approximately 12 rpm on a rotator (Scientific Instruments, Inc., St. Lake Worth, Fla.). CFU were counted at 0, 15, 30, and 60 min by a standard dilution and pour plate method. In experiments reported here, the macrophages were free of contamination by B. bronchiseptica.

Results

Uptake of Radioactivity by Macrophages. Fig. 1 illustrates an experiment in which radiolabeled, killed streptococci of type Ia, after exposure to unabsorbed rabbit sera, were incubated with coverslip cultures of alveolar macrophages. There was progressive and significant uptake of counts per minute by the cultures when the bacteria had been incubated with unheated or heated, unabsorbed serum to type Ia, no significant uptake after exposure to NRS or antiserum to types II and III, and minimal uptake after incubation with unabsorbed type Ib serum. Moreover, coverslips without macrophages did not accumulate radioactivity when incubated with labeled bacteria exposed to homologous antiserum; this last control was regularly included in all experiments but does not appear in subsequent figures.

When stained coverslips were examined under light microscopy, macrophages that had been incubated with streptococci exposed to specific antiserum appeared to contain numerous cocci (Fig. 2 A), but cultures incubated with bacteria treated with NRS did not (Fig. 2 B).

Evidence that Uptake of Radioactivity was a Quantitative Measurement of Phagocytosis. The attachment of complexes of labeled streptococci and immunoglobulin G antibody to Fc binding sites on the membranes of macrophages without significant ingestion of bacteria could result in the uptake of counts per
minute by coverslip cultures and might be difficult to exclude with certainty by light microscopy. Therefore, the following experiments were conducted.

After brief incubation at 37°C, appropriate mixtures of labeled streptococci and specific antiserum were chilled and added to macrophage cultures in Leighton tubes which rested on an ice slurry. These were rocked alongside unchilled tubes in the 37°C incubator. Chilling is known to inhibit ingestion but not attachment of immune complexes to macrophage membranes (18). As Fig. 3 shows, this procedure reduced the uptake of radioactivity by over 90% of the uptake at 37°C. When the chilled cultures were again warmed (Fig. 3, arrow), the usual uptake of counts per minute occurred over the next 2 h, indicating that inhibition by cold was reversible. Moreover, microscopic examination of phagocytic mixtures which had been rocked on ice for 2 h suggested considerable peripheral accumulation but little interiorization of bacteria (Fig. 2 C). These data indicate that the uptake of radioactivity was largely due to phagocytosis of streptococci.

Additional, indirect evidence for phagocytosis was provided by studies of
IMMUNITY TO GROUP B STREPTOCOCCI

Fig. 2. Photomicrographs of cultured macrophages after incubation with type Ia streptococci. (A) Incubation at 37°C with streptococci preincubated with type Ia serum and (B) NRS. (C) Incubation on ice with streptococci preincubated with type Ia serum. ×950.

Fig. 3. Uptake by macrophage cultures of radiolabeled type Ia streptococci at 37°C (broken lines) and on ice (solid line). The arrow indicates point at which chilled cultures were returned to 37°C environment.

streptococcal killing. It was necessary to perform these experiments with freshly collected macrophages in suspension, rather than with cultured macrophages. Nevertheless, as shown in Fig. 4, approximately 90% of the original bacterial inoculum was killed by suspended macrophages in the presence of the specific, homologous antiserum which also promoted maximal uptake of counts per minute by cultured macrophages.

Opsonization and Phagocytosis of Type Ia (Fig. 5). The uptake of radioactivity by macrophages was maximal after preincubation of type Ia streptococci with unabsorbed homologous serum, insignificant after incubation with NRS or
unabsorbed sera to types II and III, and intermediate after incubation with unabsorbed type Ib serum (Fig. 5 A). The effect of type Ia serum was apparently related to antibody to IaCHO, since it was unaffected by absorption with type Ib cells, but completely inactivated by absorption with cells of type Ia or type Ic, both of which contain IaCHO (Fig. 5 A). Similarly, the maximal opsonic activity of type Ic serum and the results of absorption with the three subtypes (Fig. 5 B) could be attributed to anti-IaCHO. On the other hand, the intermediate effect of type Ib serum, which was completely absorbed by cells of type Ia, Ib, or Ic, was apparently due to antibody to IabcCHO (Fig. 5 A).

**Opsonization and Phagocytosis of Type Ib (Fig. 6).** The finding with type Ib complemented the previous observations with type Ia streptococci in that phagocytosis was greatest after opsonization with homologous antiserum, negligible with NRS or unabsorbed serum to type II or type III, and moderate with unabsorbed type Ia serum (Fig. 6 A). The absorption studies shown in Fig. 6 B indicated that the strong homologous reaction was related to antibody to IbCHO and the moderate opsonic activity of type Ia serum to anti-IabcCHO. However, the effect of unabsorbed type Ic antiserum (Fig. 6 C), resulting in phagocytic uptake equal to that produced by type Ib serum, was largely attributable to antibody to Ibc protein, since it was completely absorbed by either type Ib or Ic. It is possible that some opsonic activity of this serum was due to anti-IabcCHO, since there was partial removal with type Ia cells.
Opsonization and Phagocytosis of Type Ib (Fig. 7). Phagocytosis of type Ib was equally significant after exposure to unabsorbed serum against type Ia, Ib, or Ic, insignificant after exposure to NRS or unabsorbed type II serum (Fig. 7A), and intermediate after exposure to unabsorbed type III serum (Fig. 7B). The absorption studies shown in Fig. 7A indicated that the activity of type Ia serum was due entirely to anti-IaCHO, and the activity of type Ib serum was due largely to anti-Ibc protein. Some of the latter may also be attributable to anti-IabcCHO since there was partial absorption by type Ia (Fig. 7A). The effect of type III serum and its absorption by three heterologous strains (Fig. 7B) were presumably related to antibody to an unidentified antigen common to all group B streptococcal serotypes.

Fig. 7C illustrates the antigen-antibody systems involved in the opsonization of type Ic by homologous antiserum. The opsonic effect of this serum was not significantly affected by absorption with streptococci of either type Ia or Ib alone. However, sequential absorption with cells of both serotypes removed all activity, as did absorption with type Ic. This serum was similarly absorbed with cells of types Ib and II to rule out nonspecific removal of essential serum opsonic factors by sequential treatment with two serotypes (Fig. 7C). Thus, antiserum to type Ic streptococci appeared to contain at least two populations of opsonic antibody, anti-IaCHO and anti-Ibc protein, either of which effected maximal phagocytosis alone.

Heat-Stability of Serum Opsonins. As shown in Fig. 1, 4, 5A, 6A, and 7A, heating of serum at 56°C for 30 min had no significant influence on the opsonic effect of sera against the homologous serotypes. In addition, maximal and
FIG. 6. Uptake by macrophage cultures of radiolabeled type Ib streptococci preincubated with various sera. (A) Unabsorbed sera only. (B and C) Absorbed and unabsorbed sera.
FIG. 7. Uptake by macrophage cultures of radiolabeled type Ic streptococci preincubated with absorbed and unabsorbed sera.
intermediate levels of opsonic activity against heterologous serotypes were not modified by heating.

Discussion

The most definitive evidence regarding immunity to the group B streptococci is derived from studies in mice of virulence and protection by rabbit antiserum. Thus, Lancefield and associates have demonstrated protection against group B streptococci by a number of specific antibodies. Anti-IaCHO was shown to be protective against type Ia infection and anti-IbCHO against type Ib (4). Anti-IabcCHO, apparently present only in certain sera, was protective for infection with types Ia, Ib, or Ic (4, 7). Recently, anti-Ibc protein was shown to be protective against types Ib and Ic (7). Antibody to type II CHO is also mouse protective for the corresponding serotype (9), but direct evidence regarding type III and the significance of the other defined antigens of the group B cell wall is lacking (7, 8). Antibody to the group B CHO is apparently not protective (7).

Inasmuch as the ultimate defense of the host against most invasive bacteria is phagocytosis, the studies reported here were focused on the in vitro interaction of bacteria, serum, and macrophages in an attempt to analyze the bacterial antigens and serum factors critical to the opsonization of group B streptococci. The assay method used here was shown to measure phagocytosis quantitatively and reproducibly and offers some advantages over other in vitro methods. The indirect bactericidal test has been used extensively for group A streptococci (19), but proved to be difficult in these studies because multiplication of the group B organisms in the blood of most human donors studied was inconsistent. Another technique (17) has been used widely and successfully to study the phagocytosis and intracellular killing of various bacteria. However, these two events are not easily separable, and results with this method were not always clear cut. This may have been the result of multiplication by extracellular bacteria, even in phagocytic mixtures in which significant ingestion and killing occurred. The use of radiolabeled, nonreplicating bacteria and cultured alveolar macrophages appeared to avoid some of these technical difficulties.

This study demonstrates the absolute requirement of specific antibody to bacterial antigens, type-specific or shared, CHO or protein, for significant phagocytosis of group B streptococci of types Ia, Ib, and Ic. It is noteworthy that the IaCHO, IbCHO, and Ibc protein antigen-antibody systems were consistently associated with significantly greater phagocytosis than the IabcCHO system. It is not clear whether the subsidiary opsonic role of the latter is simply the result of smaller amounts of cross-reacting anti-IabcCHO or is inherent in the structure, quantity, location, or steric arrangement in the bacterial cell wall of IabcCHO in relation to other surface antigens.

Type Ic streptococci differ from types Ia and Ib in the possession of two antigens of major importance in opsonization. Maximal phagocytosis of type Ic followed treatment with the homologous serum, as well as with type Ia serum (presumably due to anti-IaCHO) or type Ib serum (presumably anti-Ibc protein). Type Ic serum appeared to contain both antibodies, since absorption with both Ia and Ib cells was required to abolish its opsonic activity.

One finding that could not be explained by the known scheme of bacterial
antigens was the intermediate opsonic activity of unabsorbed type III serum on type Ic streptococci. Although the Ibc protein is found sometimes in type II (20) and rarely in type III (J. Jelinkova, personal communication), anti-Ibc protein, as the source of this activity, was excluded by absorption with any of the group B serotypes. Therefore, this serum was opsonic for type Ic apparently because of antibody to an unidentified antigen common to all prototype strains.

The heat stability of rabbit serum opsonins for group B streptococci was striking and is of interest in view of the opsonic requirements for group A streptococci which, in the presence of either human antibody or hyperimmune rabbit serum, include complement and possibly other heat labile serum factors (21-23). In addition, available data suggest that the opsonic activity for group B streptococci present in some human sera is heat labile but not clearly related to complement components (24, 25).

The state of human immunity to the group B streptococci is largely unexplored and is a question of potential significance, particularly in pregnancy and the newborn period. In a study limited to a single group B strain of unspecified serotype, Dossett et al. demonstrated low but comparable levels of opsonic activity in maternal and cord sera (26). Basing their conclusions upon microscopic examination of neutrophiles after incubation with group B streptococci and serum, Klesius et al. reported that parturient women and their infants have serum opsonins against types Ib, Ic, II, and III, but not against type Ia streptococci (24). There is a need to confirm and extend these observations as a possible means of prospectively identifying pregnancies and newborn infants at special risk of serious infection with the group B streptococci.

Summary

The opsonization and phagocytosis of group B streptococci of types Ia, Ib, and Ic were studied in vitro by measuring the uptake of radioactivity by coverslip cultures of rabbit alveolar macrophages during incubation with radiolabeled, nonviable bacteria which had been exposed to rabbit serum. The uptake of counts per minute was quantitative, reproducible, and reversibly inhibited by cold, indicating that it was largely a measurement of phagocytic ingestion rather than of attachment of bacteria-immunoglobulin complexes to macrophage membranes. Moreover, suspended macrophages killed approximately 90% of viable streptococci in the presence of specific antiserum.

The opsonic activity of immune serum was heat stable, and phagocytosis of streptococci was insignificant after incubation with normal serum and antiserum to some heterologous group B streptococci. By absorption studies, it was possible to identify the effect of antibodies to specific bacterial antigens. Phagocytosis of streptococci containing the corresponding antigens was maximal after opsonization with homologous or heterologous sera containing antibody to IaCHO, IbCHO, or Ibc protein. Phagocytosis of all three serotypes was intermediate when opsonization could be attributed to anti-IabcCHO. The opsonization of a specific group B streptococcus is complex and may involve two or more antigen-antibody systems.

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IMMUNITY TO GROUP B STREPTOCOCCI


