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Hyaluronate Capsule Prevents Attachment of Group A Streptococci to Mouse Peritoneal Macrophages

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The antiphagocytic properties of the streptococcal hyaluronic acid capsule were explored in a system in which binding of the organism to the phagocyte, but not ingestion, could occur. The capsule was found to be highly effective in preventing attachment of two strains of group A streptococci to mouse peritoneal macrophages. Variation in attachment with phase of growth in broth culture (low in early log phase, high in late-log phase and thereafter) could be accounted for by production and subsequent loss of capsule. Hyaluronidase treatment removed the capsule and increased adherence; treatment with proteolytic enzymes removed M protein and decreased resistance to phagocytosis in whole human blood but did not remove the capsule or increase adherence to the mouse peritoneal macrophages.

Two surface structures of *Streptococcus pyogenes* have been identified as virulence factors in human and experimental murine infections: M protein and the hyaluronic acid capsule, both of which protect the organism from ingestion by phagocytes in vitro (10, 16). M protein appears to prevent in some manner the activation of the alternate pathway of complement (4, 22), which is required for the efficient phagocytosis of streptococci lacking M protein (22). The hyaluronate capsule, like other polysaccharide capsules, is assumed to be a protective coating that impedes access of the phagocyte to recognizable structures on the cell wall (10, 16), but its antiphagocytic properties have not been investigated in detail.

Recent reviews have emphasized that phagocytosis is a multistep process: recognition (attachment of particle to phagocyte, possibly to specific receptors), internalization by means of phagosome formation, fusion of phagosome and lysosome, and killing. Resistance to phagocytosis can be expressed at any of these steps (5, 28, 30). With regard to the recognition step, one might reasonably expect that polysaccharide capsules, with their high negative charge and hydrophilicity (11, 31), would not readily attach to mammalian cell membranes in the absence of specific receptors for their polysaccharide constituents. In studies of pneumococci and group A streptococci, for example, encapsulation interfered with adherence of the organisms to epithelial cells (1, 27). Little work on the attachment of encapsulated bacteria to phagocytes has been reported, perhaps because of the difficulty of separating attachment from ingestion; but a recent study of *Escherichia coli* showed that encapsulated organisms would adhere to phagocytes only if attached to the cell membrane with concanavalin A, and that once so attached, the organisms were not ingested in the absence of antibody and complement (14).

In this report, we employ a serum-free incubation medium at 4°C (21, 24) to prevent internalization of attached bacteria and we show that the hyaluronic acid capsule prevents attachment of group A streptococci to a phagocytic cell, the mouse peritoneal macrophage.

MATERIALS AND METHODS

Streptococci. A type 5 strain (Chile) of group A streptococcus originally isolated from a patient with rheumatic fever and subsequently from the throat of a laboratory technician with exudative pharyngitis and a type 24 strain (Vaughan) isolated from a patient who developed rheumatic fever were used in these studies. The type 24 organisms were frequently passaged through mice to promote optimal production of hyaluronate capsules and M protein (16). This strain was highly mouse-virulent (100% lethal dose, <10 colony-forming units); the type 5 strain, although rich in M protein as shown by opsonophagocytic tests in human blood, was not virulent for mice (50% lethal dose, 1.3 $\times 10^6$ colony-forming units, calculated by the method of Reed and Muensch [25]). The encapsulated and M protein-rich bacteria were grown to log phase in Todd-Hewitt broth (Difco Laboratories) supplemented with 20% rabbit serum (GIBCO Laboratories) and stored at −80°C until use, at which time an aliquot was grown overnight in unsupplemented Todd-Hewitt broth; the overnight culture was used to start the experimental culture. Organisms subcultured 1:100 in fresh Todd-Hewitt broth were grown to the desired phase, chilled to 4°C, washed twice, and finally suspended in cold

0.15 M NaCl–0.02 M phosphate at pH 7.35 (phosphate-buffered saline [PBS]) to an optical density corresponding to 10^9 individual organisms (not chains) per ml as determined by Petroff-Hausser chamber counts. Streptococcal chain length after washing averaged 2 to 3 cells.

Capsules. Capsule thickness was assessed by examining India ink preparations under a bright-field microscope and is expressed as the number of bacterial cell radii. Capsules designated "±" were less than one bacterial radius in thickness but larger than the diffraction halo that surrounded hyaluronidase-treated organisms.

Enzyme treatment of streptococci. For enzymic removal of the capsule, organisms were treated with 24 U of bovine testicular hyaluronidase (type 4, Sigma Chemical Co.) per ml at pH 7.4 for 1 h (2). Proteolytic treatment with 0.1 mg of bovine pancreatic trypsin (type 11, Sigma Chemical Co.) per ml was carried out in 0.1 M phosphate buffer at pH 8.0 for 60 min at 37°C, and then terminated with lima bean trypsin inhibitor (Worthington Diagnostics). Gentle treatment with 20 μg of dilute pepsin (Worthington Diagnostics) per ml was carried out in 0.1 M phosphate buffer at pH 5.8 for 20 min at 37°C. The reaction was terminated by raising the pH to 7.5 with 1 N NaOH. After enzyme treatment, the organisms were chilled and washed in cold PBS. For experiments involving hyaluronidase treatment only, live organisms were treated with enzyme and kept at 4°C thereafter, except as noted. For experiments involving sequential treatment of organisms with hyaluronidase and a protease (or control buffer), ultraviolet light-killed organisms were used (33) to prevent regeneration during the second treatment of surface components removed by the first.

Mouse peritoneal macrophage monolayers. For each experiment, an adult female Swiss mouse was killed by cervical dislocation, and the peritoneal cavity was rinsed with 5 ml of PBS (21). The cell suspension was adjusted to 2×10^6 cells per ml by hemocytometer count. Drops of cell suspension (200 μl) were spread on glass cover slips (22 by 22 mm^2) in 35-mm tissue culture dishes and incubated at room temperature for 30 min to allow the macrophages to settle and adhere. The monolayers were then washed three times in PBS and chilled to 4°C, taking care to keep them wet.

Macrophage adherence tests. Washed monolayers were exposed to streptococcal suspensions in various dilutions for 45 min at 4°C with horizontal swirling at 40 to 50 rpm. Capsule size remained constant throughout incubation. Trypan blue exclusion by the macrophages was always >98% at the end of incubation. The monolayers were washed by allowing a stream of cold PBS to flow over them while they were aspirated from the other side, drained briefly, fixed with methanol, and stained with Giemsa. A total of 100 to 400 macrophages was counted, noting the percentage with attached bacteria and the number of attached bacteria per cell. All counts were performed by one person, with the slide labels covered with tape and coded. Results are expressed as total number of attached bacteria divided by total number of macrophages counted (number of attached bacteria per cell).

Opsonophagocytic tests. For opsonophagocytic tests (2), a standard suspension of log phase, ultraviolet light-killed organisms in 50 μl of PBS was opsonized with 100 μl of normal rabbit serum (GIBCO Laboratories) or rabbit antiserum to purified homologous M protein (3) for 15 min at 0°C, then mixed with 0.35 ml of fresh nonimmune human blood containing heparin (10 U/ml) and incubated at 37°C with end-over-end rotation at 8 rpm. Smears were prepared at 15, 30, and 45 min of incubation and stained with Wright stain. A total of 100 polymorphonuclear leukocytes was counted, noting the number with bacteria associated (percent phagocytosis); no attempt was made to distinguish attached from ingested bacteria.

Electron microscopy. Electron microscopy was performed to assess the effect of enzyme treatments on the surface fibrillae of the streptococci (2). Enzyme-treated streptococci were fixed for 4 h in ice-cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4, washed twice in the same buffer without glutaraldehyde, postfixed for 1 h in 1.0% osmium tetroxide, dehydrated stepwise in increasing concentrations (50, 75, 90 and 100%) of ethanol, and embedded in Spurr medium (Polyscience).

RESULTS

Under conditions chosen for maximal adherence and minimal ingestion, i.e., heavy suspensions of streptococci in a serum-free, divalent cation-free system at 4°C, stationary-phase type 5 streptococci adhered to mouse peritoneal macrophages in a concentration-dependent manner (Fig. 1). Adherence reached a plateau after 30 to 40 min of incubation (Fig. 2). These organisms were unencapsulated, and attachment was not increased by pretreating them with hyaluronidase. When adherence was determined at successive points on the growth curve, early log phase, encapsulated streptococci adhered minimally, regardless of the size of the capsule. Adherence remained low even after the capsule had apparently disappeared, but increased rapidly in late-log phase to a plateau (Fig. 3).

To see whether the capsule was, in fact, responsible for the poor adherence of the early log-phase streptococci, the organisms were tested after decapsulation with hyaluronidase. The bacteria were prepared by resuspending only the topmost slimy layer of the washed pellet to make sure that 100% of the organisms were encapsulated. Hyaluronidase treatment of the encapsulated log-phase organisms removed the capsule and increased adherence from virtually nil (0.1 attached organisms per cell at the highest concentration of streptococci) to the high levels obtained with stationary-phase organisms (Fig. 4). When the hyaluronidase-treated organisms were washed and placed in fresh broth at 37°C, most (about two-thirds) grew new capsules within 15 min, and attach-

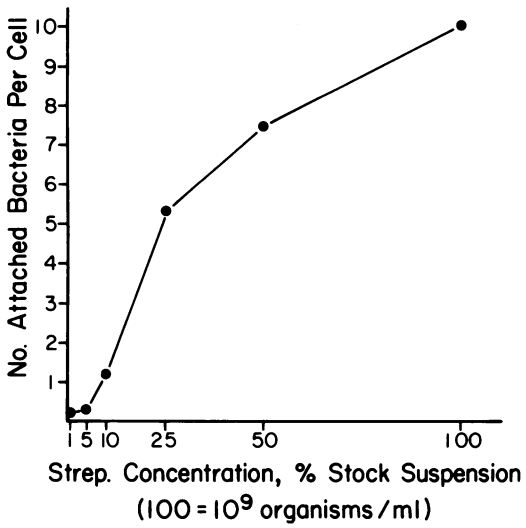

FIG. 1. *Concentration-dependent adherence of stationary-phase, unencapsulated type 5 streptococci to mouse peritoneal macrophages in a serum-free system at 4°C.*

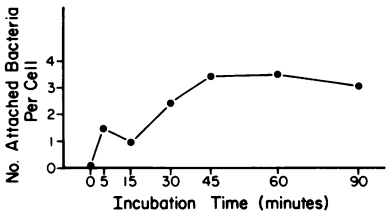

FIG. 2. *Time dependence of streptococcal adherence. Streptococcal concentration is 0.5 × 10⁹ organisms per ml.*

ment fell to low levels. On continued incubation for 2 h, the capsules disappeared, and attachment increased to the level obtained after hyaluronidase treatment.

To exclude the possibility that release of M protein or other surface proteins in the process of decapsulation (20) was contributing to the effect of hyaluronidase, the effect of proteolytic treatment on adherence of encapsulated organisms was examined. For these experiments, a strain of type 24 was used as the type 5 organisms tended to decapsulate spontaneously in the proteolysis reaction buffer at 37°C, even after killing by ultraviolet light. (The destruction of hyaluronic acid by some strains of killed log-phase streptococci has been observed previously [23].) Log-phase, encapsulated type 24 streptococci were killed by exposure to ultraviolet light, which did not alter the capsule, the surface fibrillae, or the ability of the organisms to resist phagocytosis, as described previously (2, 33). Two proteolytic treatments were used: trypsin, which removes the surface fibrillae, and dilute pepsin at suboptimal pH, which does not (2), although in this study pepsin-treated fibrillae were sparse and somewhat shortened. Complete removal of functional M protein by both enzymes was confirmed by phagocytosis tests in whole human blood, which showed loss of resistance to phagocytosis and no increase in phagocytic rate after opsonization with antibody to purified type 24 M protein (Fig. 5). Neither pepsin nor trypsin treatment removed the capsule (9, 20), and adherence remained low. Proteolytic treatment of hyaluronidase-treated organisms did not reduce their adhering ability; pepsin treatment, in fact, increased it (Fig. 6).

DISCUSSION

This study was designed to focus on the attachment step of phagocytosis by employing experimental conditions in which ingestion would not occur. Although the method we used for measuring bacterial attachment to phagocytes—counting the number of cell-associated streptococci by light microscopy—does not in itself permit the distinction between attachment and ingestion, the experiments were carried out in a serum-free medium at 4°C, conditions in which phagocytosis occurs very slowly, if at all (24, 28). Accordingly, we conclude that the hyaluronate capsule is highly effective in preventing attachment of streptococci to mouse peritoneal macrophages. Furthermore, the large increase in adhering ability that occurred during the transition from the logarithmic to the stationary phase of growth was apparently due to spontaneous decapsulation, since treatment of early log-phase, encapsulated organisms with hyaluronidase, but not proteolytic enzymes, increased attachment to the level observed in the stationary phase. The low level of attachment at the 0.125 optical density point in Fig. 3 was, therefore, probably due to a capsule too thin to be detected by India ink preparation, which is a technique of limited sensitivity (6). Spontaneous loss of capsule by group A streptococci during growth in broth culture has been observed frequently (8, 17-19). The process appears to be enzymic (17, 23, 32) but is not necessarily accompanied by release of hyaluronidase into the culture medium (23).

In studies of the adherence of group A streptococci of types 1, 3, 12, and 56 to human oral or pharyngeal epithelial cells (1, 7), the relation of phase of growth to adherence followed a pattern similar to that reported here, that is, low adherence in log phase, increasing markedly by sta-

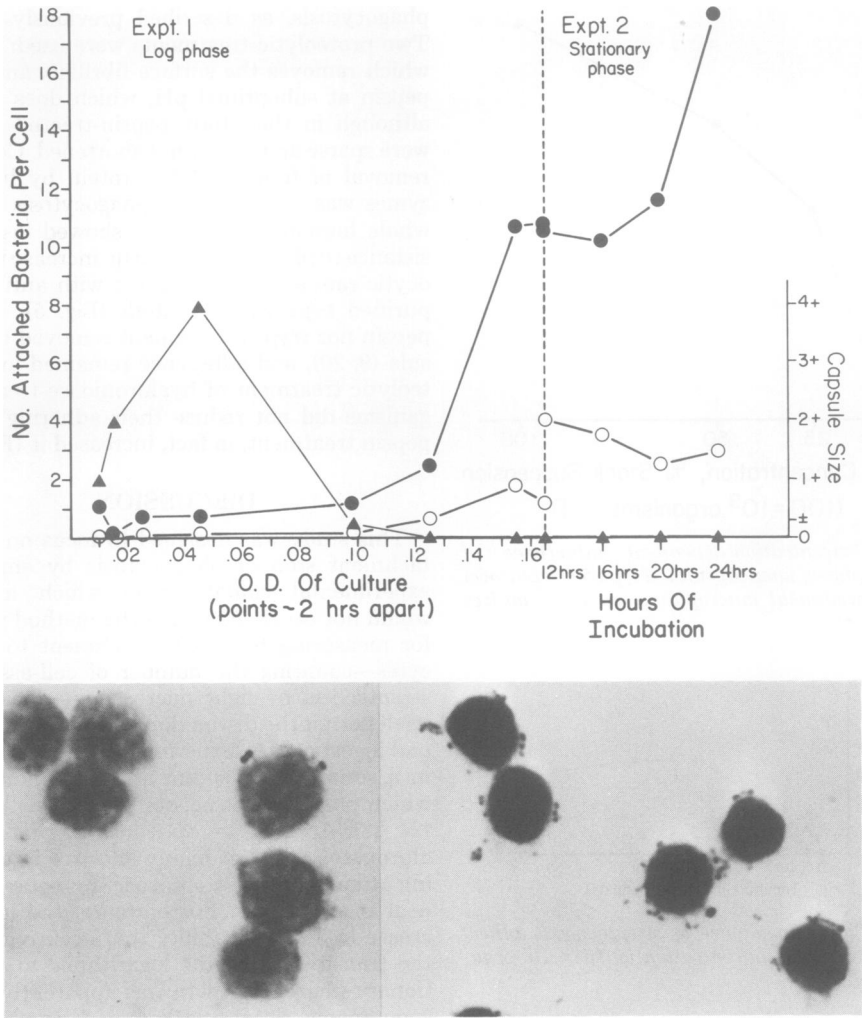

FIG. 3. *Mouse macrophage adherence and capsule size of type 5 streptococci at various stages of growth. Symbols: ●, attachment of organisms at a concentration of 10^9 per ml; ○, a 10-fold dilution of the same streptococcal suspension; ▲, average thickness of the capsule. O.D., optical density at 530 nm, 8-mm light path. Photomicrographs of macrophage monolayers from the 0.01-O.D. point (left) and the 24-h point (right) are shown. (The monolayer on the right was drained for a shorter period before fixation, accounting for the more compact appearance of the macrophages.)*

tionary phase. The time course of encapsulation was not followed in those studies, but in one of them (1) it was noted that hyaluronidase treatment of an encapsulated strain increased its adherence to pharyngeal epithelial cells. In view of our results, we suspect that production and subsequent loss of hyaluronate capsule during growth in broth culture account for the pattern of adherence observed by those investigators. The implication of the finding that the capsule interferes with adherence to epithelial cells is that encapsulation might be detrimental to survival of streptococci on the pharynx, since replication of the organisms on the mucosa requires attachment to the epithelium (7). We noted that streptococci can synthesize and destroy their capsules rapidly (Fig. 4). It is possible, therefore, that streptococci are unencapsulated during the initial phase of colonization and tissue invasion, forming capsules only when they encounter an inflammatory exudate or tissue infiltrate.

In this study we were not primarily concerned with the nature of the binding of the unencapsulated streptococci to the macrophages, but several observations are worth mentioning. The attachment resembled that of a weakly bound

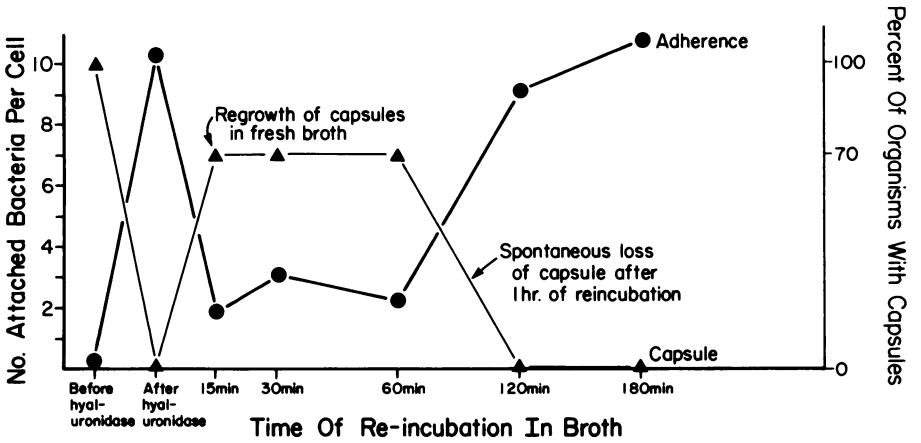

FIG. 4. *Effect of hyaluronidase treatment on encapsulation and macrophage adherence of log-phase streptococci. On reincubation in fresh broth, most of the organisms grew new capsules, and then lost them spontaneously. Symbols: ●, attachment of organisms at a concentration of 10^9 per ml; ▲, percent of organisms in the suspension having a capsule; capsule thickness was 1+.*

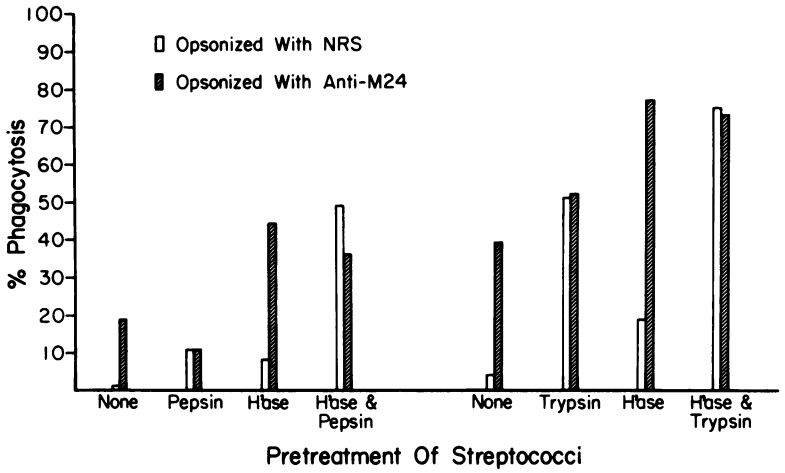

FIG. 5. *Effect of enzyme treatments on resistance of ultraviolet light-killed type 24 streptococci to phagocytosis in whole human blood (two experiments). In the trypsin experiment a slightly larger inoculum was used. NRS, normal rabbit serum; h'ase, hyaluronidase.*

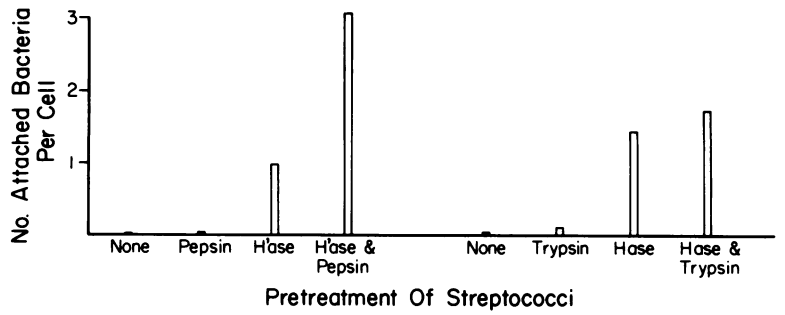

FIG. 6. *Effect of enzyme treatments on adherence of type 24 streptococci to mouse peritoneal macrophages (same organisms as those in Fig. 5). H'ase, hyaluronidase.*

ligand to a receptor, in that only a small fraction of the bacteria present became attached at subsaturating concentrations, but this observation does not necessarily implicate a specific ligand-receptor interaction in the attachment process. We were unable to "saturate" the macrophages because the streptococci agglutinated at high concentrations. In previous investigations in our laboratory (2), adherence of M24 streptococci to oral epithelial cells was abolished by treatment of the cocci with trypsin. In this study, trypsin treatment did not diminish attachment to the mouse macrophages even though it removed all of the fibrillae. Presumably the macrophage, unlike the epithelial cell, can recognize cell wall structures unmasked by trypsin treatment.

An interesting finding of this study was that under the incubation conditions used, the ability of the capsule to prevent adherence was unrelated to the mouse virulence of the strain and was not affected by enzymic removal of M protein. Moreover, when care was taken to be sure that only encapsulated organisms were present in the suspension, attachment was negligible even at concentrations of streptococci (10^9/ml) far exceeding those customarily employed in in vitro phagocytosis tests or mouse virulence assays. In contrast, previous investigations of the relative contributions of M protein and capsule to virulence and to resistance to phagocytosis in vitro have shown, in general, that the capsule is considerably less important than M protein in both types of experiment, and, unlike M protein, is not necessary for virulence (9, 12, 13, 15, 16, 20, 26). Of course, ingestion rates (as measured by phagocytosis tests) and adherence affinities are not comparable quantities, and in principle this "discrepancy" could be explained if ingestion were so rapid that only the briefest contact between bacterium and phagocyte membrane sufficed for enclosure of the bacterium in a phagosome. An alternative explanation seems more likely, however, namely that under physiologic conditions, factors deriving either from the phagocyte or the medium serve in some way to defeat the capsule. The existence in human serum of one or more heat-labile substances (other than complement) directed specifically at the streptococcal capsule has been proposed by several investigators on the basis that, in general, only human serum or plasma supports the phagocytosis of encapsulated streptococci (12, 29). The nature and mode of action of these "coöpsonins" and whether anything comparable exists in the mouse peritoneal cavity are not known. A phagocyte adherence assay such as that reported here may prove useful for the study of these and other host defenses against polysaccharide capsules.

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