Valentin-Weigand, P., Benkel, P., Rohde, M., Chhatwal, G.S.

Entry and Intracellular Survival of Group B Streptococci in J774 Macrophages

PETER VALENTIN-WEIGAND, PETRA BENKEL, MANFRED ROHDE, AND GURSHARAN S. CHHATWAL

Department of Microbiology, GBF-National Research Centre for Biotechnology, Braunschweig, Germany

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The mouse macrophage-like cell line J774 was used to analyze opsonin-independent entry and survival of group B streptococci (GBS). Efficient entry of GBS in J774 cells occurred within 5 min postinfection, and streptococci persisted intracellularly without loss of viability for at least 8 h. At 24 h postinfection, 30% of the total intracellular GBS was recovered from macrophages. Inhibition studies using different biochemical modulators of cellular functions showed that bacterial entry seemed to involve nonglycosylated J774 surface structures different from known receptors such as fibronectin-binding integrins. Internalization of GBS by J774 cells occurred by a microfilament-dependent phagocytosis-like process also involving participation of receptor-mediated endocytosis. Prior opsonization of GBS with human serum containing anti-GBS antibodies did not affect bacterial entry but significantly reduced the intracellular survival of GBS. Transmission electron microscopic analysis confirmed these findings and demonstrated that both opsonized and nonopsonized bacteria were contained within phagosomes during the whole infection period. Transmission electron microscopy further revealed that decreased intracellular survival rates of opsonized GBS appeared to be due to increased lysosomal activities of the macrophages. These results suggest that in the absence of opsonins, GBS are able to enter and persist efficiently in macrophages by evading intracellular antibacterial activities commonly associated with opsonin-mediated uptake.

Group B streptococci (GBS) are a major cause of pneumonia, sepsis, and meningitis in neonates (5). The main route of infection is assumed to be aspiration of amniotic fluid or vaginal contents containing GBS by the neonate during parturition and subsequent streptococcal colonization of the upper and lower respiratory epithelium (4, 5). Pneumonia results from local infections, whereas sepsis and meningitis may be due to the spread of bacteria followed by systemic infection (4). This indicates that GBS can avoid host defense mechanisms and rapidly proliferate in fetal airways. Furthermore, GBS probably can gain access to the bloodstream and maintain a high level of bacteremia, leading to the onset of bacterial meningitis (11, 24). Invasion and intracellular survival of GBS in host cells thus represent important pathogenicity mechanisms in invasive GBS infections.

Two main cell types, respiratory epithelial cells and resident alveolar macrophages, are encountered by GBS infecting the lung (2, 14, 35). The former are the first barrier for streptococcal transcytosis into deeper tissues and to the bloodstream. The recently reported ability of GBS to enter and survive in respiratory epithelial cells may represent a mechanism by which these bacteria gain access to the blood circulation system (30, 31, 40). Pulmonary alveolar macrophages, on the other hand, can play a crucial role as a first line of local host defense, since they can readily kill GBS opsonized by type-specific antibodies and/or complement (9, 34, 36). However, in the neonatal lung resident alveolar macrophages are often found in reduced numbers and are probably defective in their antibacterial activity (3, 34, 35). In addition, local opsonin concentrations in the lung are relatively low and may not be sufficient to facilitate effective killing of bacteria (14, 29). Under such conditions, entry and survival of GBS in macrophages could play an important role in the course of invasive GBS infections. Most studies on interactions of GBS with macrophages have focused on the role of antibodies and complement in phagocytic killing (21, 34–36). Recently, opsonin-independent phagocytosis of GBS has been reported and shown to be mediated by complement receptor type 3 (3). However, little is known about the pathogenic significance of opsonin-independent invasion of GBS in macrophages and the mechanisms involved in uptake and intracellular survival. We, therefore, investigated the interactions of GBS with macrophages in order to compare the uptake and survival of nonopsonized and opsonized GBS. Results from this study suggest that GBS can enter macrophages in the absence of opsonins via a mechanism involving both microfilament-dependent phagocytosis and receptor-mediated endocytosis. Furthermore, it is shown that opsonin-independent entry of GBS results in intracellular survival that is significantly enhanced compared with that resulting from opsonin-mediated entry, probably because entry in the absence of opsonins leads to a decreased activation of the macrophage’s antimicrobial activity.

MATERIALS AND METHODS

Bacteria and cell culture. GBS strain 6313 is a serotype III clinical isolate obtained from an infected neonate. Bacteria were grown in Todd-Hewitt broth (Oxoid, Basingstoke, England) and maintained as stock cultures in 50% glycerol-Todd-Hewitt broth at −70°C. Working cultures were made by growing 0.5 ml of the frozen stocks in 50 ml of Todd-Hewitt broth to late log phase (optical density of 0.7 to 0.8 at 600 nm). The bacteria were washed twice in phosphate-buffered saline (PBS), pH 7.4, and adjusted photometrically (600 nm) to approximately 5 × 10^8 CFU/ml.

The murine macrophage-like cell line J774 (J774 A.1; ATCC TIB 67) was grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Life Technologies, Eggenstein, Germany) supplemented with 10% fetal calf serum (Sigma Chemical Co., Deisenhofen, Germany), 5 mM glutamine, penicillin G (100 IU/ml), and streptomycin (100 μg/ml) (all from GIBCO) at 37°C under 8% CO2.

Invasion assays. For invasion assays, J774 cells were transferred to 24-well tissue culture plates (Nunc, Roskilde, Denmark) at approximately 2 × 10^5 cells
per well and, after overnight cultivation, were washed twice in HEPES (N-2hydroxyethylpiperazine-N’-2-ethanesulfonic acid)-buffered DMEM without supplements.

The invasion of GBS was assayed as described by Rubens et al. (31) with some minor modifications (40). Cell monolayers were infected with streptococci by adding 1.25 × 10^5 bacteria per well (in HEPES-buffered DMEM). Streptococci were allowed to invade J774 cells for 5 to 120 min before penicillin (5 μg/ml) and gentamicin (100 μg/ml) were added to kill extracellular bacteria. These antibiotics do not penetrate eucaryotic cells under these conditions (8, 43). Cells were then lysed with 0.025% Triton X-100, and viable intracellular streptococci determined by quantitative plating of serial dilutions of the lysates on Todd-Hewitt broth-agar. Each test was done in triplicate, and the number of CFU recovered per well (mean ± standard deviation) was determined. Wells without macrophages served as negative controls. Survival experiments were done as described in the standard protocol, except that antibiotic-containing medium was left on infected cells up to 48 h postinfection.

For experiments with opsonized bacteria, streptococci were preincubated for 30 min at 37°C with fresh human serum (obtained from healthy volunteers) containing antibodies to GBS as revealed from dot blot reactivities obtained by using whole cells from GBS strain 6313. GBS-negative human serum was used as a control. Additionally, we used a polyclonal antiserum to GBS strain 6313 which had been raised in rabbits by using whole heat-inactivated bacteria. Immunglobulin G (IgG) fractions were isolated from human and rabbit sera by affinity chromatography on protein A-Sepharose (Pharmacia, Sweden) followed by protein A-Sepharose bound with rabbit antimouse IgG (Jackson). IgG fractions were isolated from human and rabbit sera by affinity chromatography on protein A-agarose (Sigma). F(ab)2 fragments were generated by pepsin digestion and separated from Fc fragments by standard procedures. Serum were used either freshly or after inactivation at 56°C for 30 min to determine the possible effects of the complement. After opsonization, the bacteria were immediately tested in invasion assays with rat peritoneal macrophages. In preliminary experiments we found that neither the immune human serum nor the rabbit antiserum caused agglutination of the streptococci.

Pretreatment of macrophages with modulating reagents. In these experiments J774 macrophages were preincubated with reagents (all purchased from Sigma) affecting different cellular functions. The following compounds were used at the indicated working concentrations: tyrophosine D (CD) (0.01 to 1 μg/ml), nocodazole (NC) (0.05 to 5 μg/ml), monodansylcadaverine (MDC) (10 μM), tunicamycin (FN) (5 μg/ml), and synthetic peptide GRGDSP (RGD) (20 μM). Macrophages were incubated with these compounds at 37°C for 30 min (CD) or 1 h (MDC, mitomycin, tunicamycin, FN, and RGD) or for 1 h on ice and subsequently for 30 min at 37°C (NC) prior to infection. Compounds were then left on the cells for the whole infection period.

Electron microscopy. For transmission electron microscopy (TEM), infected cells were fixed for 1 h on ice with a solution containing 3% glutaraldehyde and 5% formaldehyde in cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M MgCl2, 0.01 M CaCl2, pH 6.9). After several washings with cacodylate buffer, cells were fixed with 1% (wt/vol) osmium tetroxide in cacodylate buffer for another hour at room temperature, washed again with cacodylate buffer, and scraped off the culture flask. Cells were centrifuged for 2 min at 2,000 × g and embedded in 1.5% agar. Small cubes of the agar were dehydrated with a graded series of acetone and embedded in Spurr resin (37), and ultrathin sections were cut with glass knives. After counterstaining with 0.5% uranyl acetate (pH 4.5) for 30 min at 40°C and with 1% lead citrate for 30 min at 20°C (Ulbricht, Lehica, Bensheim, Germany), sections were examined at calibrated magnifications with a Zeiss transmission electron microscope (EM 910) at an acceleration voltage of 80 kV. Lysosomal vesicles were counted in 30 sections of macrophages with similar section planes (i.e., in every section the nucleus was clearly visible). Lysosomes were identified by their characteristic appearance as round vesicles surrounding or fusing with phagosomes (see Fig. 5) as well as by their specific staining in immunoelectron microscopy using a monocolonal antibody (MAC-3) against a glycoprotein of the lysosomal membrane as described previously (18).

DIF microscopy. Double immunofluorescence (DIF) was performed essentially as described by Heesemann and Laufs (20). All dilutions were made in PBS containing 10% fetal calf serum, and all incubations were done for 45 min at 37°C. Infected cells grown on round glass coverslips were fixed with 3.7% formaldehyde, washed with PBS, and incubated with 1:100-diluted polyclonal antibodies to GBS strain 6313(pAb63). After three washes with PBS, fluorescein isothiocyanate-labeled secondary antibody to rabbit IgG (diluted 1:50, Sigma) was used to detect attached extracellular streptococci. A second incubation with pAb63 antibodies followed the permeabilization of infected cells with 0.1% Triton X-100 in PBS. Washed cells were then probed with a tetramethyl-rhodamine isothiocyanate-labeled antibody to rabbit IgG (diluted 1:50, Sigma) bound to the intracellular streptococci. Samples were washed again, mounted with Moviol (Merck, Darmstadt, Germany), for microscopy, and examined with a Zeiss Axioscope microscope equipped for epifluorescence detection.

RESULTS

Kinetics of uptake and survival of GBS in the absence of opsonins. To monitor the uptake process quantitatively, streptococci were allowed to invade J774 cells for 5 to 120 min, and after antibiotic killing of extracellular bacteria, internalized viable streptococci were determined by quantitative platings. These experiments revealed that the internalization of GBS by J774 macrophages was a rapid process: after a 5-min invasion period almost 50% of the inoculum could be recovered from antibiotic-treated macrophages. At 120 min postinfection, intracellular recoverable CFU only slightly increased to ca. 60%, indicating that uptake was saturated within a very short period of time (Fig. 1, left-hand part). DIF experiments done without antibiotic treatments confirmed this conclusion. A high number of intracellular streptococci (10 to 25 per macrophage) was detected within the first 15 min of invasion, and at 2 h postinfection this number had only slightly increased to 25 to 35 per macrophage. Preliminary experiments revealed that replication of extracellular streptococci during the infection period could be neglected, since growth in the culture medium with and without cells was not significant.

To monitor intracellular survival for a longer period of time, invasion was done for 2 h to get optimal internalization and then samples were taken at different time points after the addition of antibiotic-containing medium. Results showed that up to 8 h postinfection intracellular numbers of viable bacteria remained almost constant. Between 8 and 24 h postinfection, a linear decrease in the number of viable intracellular bacteria was observed, decreasing by 24 h to 71% of that observed at 2 h. Between 24 and 48 h postinfection, intracellular streptococci seemed to be degraded rapidly (Fig. 1, right-hand part). The time course of invasion was also analyzed qualitatively by TEM, which confirmed the rapid uptake process. At 5 min postinfection, many intracellular bacteria could be detected. The uptake process was accompanied by the formation of phagosomes in which streptococci seemed to reside for the whole infection process. At early infection stages these phagosomes appeared rather small, containing only one or two bacteria (Fig. 2A and B), whereas later much larger phagosomes, containing five or more bacteria, were seen (Fig. 2C and D). This could have been due to intracellular replication or fusion.

FIG. 1. Kinetics of GBS entry and survival in J774 macrophages. The left-hand part of the figure shows the results of experiments carried out to monitor bacterial entry. For this, macrophages were infected with GBS strain 6313 and streptococci were allowed to invade the cells for the indicated times (5 min to 2 h). Then, antibiotics were added to kill extracellular bacteria and the numbers of internalized streptococci were determined by quantitative plating. The right-hand part of the figure shows the results of survival experiments in which streptococci were allowed to invade the cells for 2 h before antibiotics were added (arrow). Numbers of viable intracellular streptococci were then determined at the indicated times postinfection (4 to 48 h). All results are expressed as CFU recovered per well (means ± standard deviations [error bars] obtained from triplicate experiments).
FIG. 2. TEM of J774 macrophages infected with GBS strain 6313. Samples were taken at 5 min (A), 20 min (B), 1 h (C), 4 h (D), 8 h (E), and 24 h (F) postinfection. Note the large number of intracellular GBS at 5 min postinfection and the increasing sizes of the vacuoles containing many intact and some degraded bacteria (arrows) at later stages. L, lysosomal vesicles. Bars represent 2 μm (A to D) and 0.5 μm (E and F).
of smaller, bacterium-containing phagosomes. The latter is more likely since it correlates with the results obtained by quantitative platings (Fig. 1). However, it has to be considered that in these experiments antibiotics were left on the macrophages and could have killed intracellular streptococci which might have been expelled into the culture medium during prolonged invasion times. At 24 h and later stages postinfection, increasing numbers of degraded streptococci within phagosomes were seen (Fig. 2E and F), confirming the data obtained from quantitative plating experiments.

Mechanisms involved in opsonin-independent invasion of GBS. The elucidation of mechanisms involved in invasion was carried out by using reagents known to modulate certain cellular functions. CD, which depolymerizes actin, thereby blocking the eucaryotic microfilament system (1, 12), inhibited uptake of GBS by 84%. This inhibition was significant even at very low concentrations (0.2 μg/ml) and was dose dependent in the range of 0.1 to 1.0 μg/ml (Fig. 3A). Higher concentrations resulted in visible morphological alterations of the cells and therefore were not considered. Significantly reduced uptake and survival were also observed with macrophages pretreated with NC or MDC, both inhibitors of receptor-mediated endocytosis, by interfering with the cell's microtubulin system and blocking of the formation of clathrin-coated pits, respectively (1, 33, 41). Inhibitions by both substances were dose-dependent and reached more than 80% with NC and 54% with MDC (Fig. 3B). Mitomycin, a reagent which arrests cells at mitosis (1), completely blocked GBS invasion in macrophages (Fig. 3B).

Treatment of macrophages with tunicamycin had no effects on GBS invasion, indicating that N glycosylation of surface receptors was not required for an effective uptake of GBS. The synthetic peptide RGD had a very weak inhibitory effect on invasion (14%), suggesting that cellular receptors involved in streptococcal internalization did not belong to the RGD-binding integrin family (22). On the other hand, fibronectin, which also carries an internal RGD sequence (22, 27), inhibited invasion by 30% (Fig. 3B). TEM and DIF analyses, however, showed that this inhibition was not due to blocking of potential receptors needed for streptococcal attachment and entry but rather due to decreased intracellular survival (data not shown).

This was confirmed by the results of quantitative determinations of lysosomal vesicles as an indication of intracellular antibacterial activity: macrophages pretreated with FN contained 166 lysosomal vesicles in 50 sections per macrophage (3.3 ± 1.3 per section), whereas in controls only 109 lysosomes in 50 sections per macrophage (2.2 ± 1.2 per section) were counted.

Invasion of nonopsonized versus opsonized GBS. GBS were opsonized with fresh normal and heat-inactivated human serum containing anti-GBS antibodies, and their abilities to invade and survive in J774 cells for different times were compared with those of nonopsonized GBS. At 15 min postinfection, the number of opsonized viable intracellular bacteria after antibiotic treatment of the macrophages was reduced by up to 40% compared with the number of nonopsonized GBS. At 2 h postinfection, the number of opsonized bacteria surviving intracellularly was reduced by 90% compared with the number of nonopsonized bacteria (Fig. 4). Comparison of GBS opsonized with normal or heat-inactivated serum revealed no significant differences (Fig. 4), indi-
Phagosome. Bars represent 0.5 μm. Panel B is greater than that in panel A. (C) A lysosomal vesicle fusing with a phagosome. Bars represent 0.5 μm.

**DISCUSSION**

Phagocytic clearance by macrophages represents a major host defense mechanism against bacteria infecting the respiratory tract. Many studies in recent years have given evidence that effective uptake and killing of GBS require opsonization by specific antibodies and/or complement (4, 9, 35). However, it is known that GBS can also be internalized by macrophages in the absence of opsonins (3, 6, 23). These studies were aimed mainly at elucidating the role of complement in phagocytic killing and promotion of inflammation, whereas the question of streptococcal survival in macrophages and the possible role of macrophages as intracellular niches has, to our knowledge, received no considerable attention.

In the present study we analyzed the entry and subsequent survival of nonopsonized and opsonized GBS in macrophages. For this we used the well-established macrophage-like mouse cell line J774, which has features similar to those of normal macrophages and has frequently been used in functional studies on the phagocytosis of GBS (16, 17, 28).

Our results show that in the absence of opsonins, GBS entered macrophages rapidly in large numbers, indicating that effective uptake was possible without involvement of antibody- or complement-mediated phagocytosis mechanisms. It is known that GBS and some other bacteria, such as *Bordetella pertussis* or *Escherichia coli*, can directly interact with β2 integrins, a family of glycosylated protein receptors found on macrophages and other phagocytes (2, 32, 44; for a review of integrins, see reference 22). Furthermore, uptake of bacteria by phagocytes has been reported to be enhanced by FN (21, 46), an adhesive glycoprotein which is also recognized by eucaryotic receptors of the integrin family and mediates adherence of streptococci to epithelial cells (38). Interactions of macrophages with FN are known to occur mainly via the amino acid sequence RGD, which is contained not only in FN but also in other adhesive glycoproteins (22, 27, 45). Thus, we used FN and a synthetic peptide, RGD, in order to block RGD-binding surface components on J774 macrophages which might be possible candidates for receptors involved in GBS uptake. We found that FN caused a 30% reduction of GBS invasion whereas RGD had no such effects. However, according to our TEM results, the inhibitory effects of FN were not due to the blocking of potential “invasin” receptors but rather a result of enhanced macrophage antibacterial activity, thereby reducing the number of intracellular surviving GBS. This interpretation is also supported by previous findings of others that the binding of FN may enhance bactericidal activities of phagocytes (21, 46), an effect which has been shown to require cross-linking of fibronectin and, thus, could not have been observed with RGD peptides (46).

In this study, we also tried to elucidate the cellular mechanisms involved in uptake of GBS. Two principle uptake mechanisms are phagocytosis and receptor-mediated endocytosis (7, 15, 41). The former is usually involved in the uptake of larger particles, such as bacteria coated with antibodies and/or complement, and is accompanied by rearrangement of the cellular actin microfilament system. Thus, phagocytosis is characterized as a process sensitive to CD4, a reagent which specifically blocks the polymerization of actin (1, 39). Our results demonstrate that opsonin-independent entry of GBS in J774 macrophages is CD sensitive and, thus, can be classified as a typical phagocytosis-like mechanism. On the other hand, we found that entry of GBS could also be inhibited by NC and MDC.
Both substances are known to inhibit receptor-mediated endocytosis, a mechanism usually involved in the cellular uptake of smaller particles (1, 41). NC interferes with the microtubulin network, and MDC blocks a transglutaminase needed for the formation of clathrin-coated pits, which are characteristic for endocytosis (15, 26). Our results, therefore, suggest that GBS entry into J774 macrophages involves both types of uptake mechanisms of the cell. It is not yet clear, however, whether receptor-mediated endocytosis is directly involved in the uptake process or whether it affects the recycling of possible invasin receptors of the macrophages (25).

GBS not only entered macrophages very efficiently but also survived intracellularly for more than 24 h, a period which in vivo would be more than sufficient to maintain a bacteremia required for developing meningitis. Long-time survival of streptococci, however, required the absence of opsonins, since pretreatments of the bacteria with fresh or heat-inactivated human serum containing antibodies to GBS decreased their intracellular survival by more than 90%. TEM analysis also revealed that after 8 h, most of the opsonized intracellular GBS were degraded whereas at the same time point postinfection almost all nonopsonized intracellular GBS appeared to be morphologically intact. The enhanced destruction of intracellular streptococci caused by preincubation with immune human serum seemed to involve Fc receptors on the macrophages, since similar effects were seen with IgG purified from the tested sera but not with respective F(ab)2 fragments.

Long-term survival in macrophages has also been observed with Streptococcus suis (42), but the mechanisms of streptococcal survival have not been identified. Since, in our studies, both opsonized and nonopsonized GBS were only detected in phagosomes, we assumed that nonopsonized GBS must have been able to evade intraphagosomal antibacterial activities. Several mechanisms, such as inhibition of phagolysosomal fusion, inhibition of the respiratory burst, resistance to lysosomal enzymes, or attenuation of phagosomal acidification (10, 13), have been reported to be used by other intracellular pathogens in order to evade intraphagosomal killing. To address this question, we determined the number of lysosomal vesicles required for phagolysosomal fusions. Our results showed significantly more lysosomal vesicles in macrophages infected with opsonized GBS, which allows the conclusion that intracellular survival of nonopsonized GBS may be due to the bacteria’s ability to inhibit phagolysosomal fusion. It may, therefore, be speculated that the reduced intracellular killing of GBS by lysosomal activities could have occurred as a result of an opsonin-independent uptake pathway, thereby leading to intracellular direction of GBS to unfused compartments as has been found to occur with other invasive pathogens (10, 13). Further studies are needed to identify the cellular and bacterial counterparts involved in this type of GBS invasion in macrophages. This would certainly help to further the understanding of the role of GBS-macrophage interactions in the infection process.

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