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Neutrophil Fc Receptor Participation in Phagocytosis of Type III Group B Streptococci

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Human peripheral blood neutrophils bear receptors for immunoglobulin G, FcRII, and FcRIII that differ structurally and functionally. We investigated the role of FcRII and FcRIII in the phagocytosis of group B streptococci (GBS) by measuring neutrophil uptake of radiolabeled type III GBS. The mean uptake of GBS opsonized with human serum containing complement and specific antibody was 76%, but when this serum was heated, the mean uptake was only 22%. A monoclonal antibody to FcRIII, Leu-11b, inhibited in a dose-dependent manner uptake of GBS opsonized with heated or intact serum to maxima of 40 and 30%, respectively. Conversely, a monoclonal antibody to FcRII, IV.3, inhibited by 77% the uptake of GBS opsonized with heated serum but had no effect when GBS was opsonized with intact serum. Leu-11b and IV.3 had an additive inhibitory effect with heated but not with intact serum. Neither monoclonal antibody inhibited the uptake of GBS opsonized with hypogammaglobulinemic serum. Therefore, FcRII is the primary mediator of the phagocytosis of GBS opsonized by antibody alone, whereas FcRIII plays a lesser role. Surprisingly, FcRII is not necessary for phagocytosis when complement is also present. FcRIII participates, to a limited extent, in phagocytosis of GBS opsonized with antibody whether or not complement is present. These findings suggest that the function of FcRII in triggering phagocytosis may be particularly important in host defense against type III GBS in the setting of complement deficiency of young infants.

Phagocytosis is a central host defense mechanism against group B streptococci (GBS), a major cause of human infections; many such infections are caused by type III GBS (4). In order to be ingested and killed efficiently by neutrophils (polymorphonuclear leukocytes [PMN]), type III GBS must be opsonized with antibody and complement (2, 13, 26). Absence of the latter drastically reduces or abolishes killing of GBS by PMN. Although not an absolute requirement, antibody maximizes phagocytosis and killing when complement also is present (10). The role of antibody cannot be underestimated, because it protects against infection when present in sufficient concentration in human sera and in experimental models of infection (5, 15).

Since most neonates without protective levels of specific antibody do not become symptomatic with GBS, and because age-related susceptibility to GBS infection is not related to development of specific antibody, other host defense factors must be in operation (4). The nature of these factors is still unclear. An important area of investigation in this respect is ligand-receptor interactions that initiate ingestion and culminate in killing by PMN of opsonized GBS. PMN receptors for both immunoglobulin G (IgG) and complement factors are known to participate in these processes. Smith et al. (27) demonstrated that monoclonal antibodies (MAb) to PMN complement receptors significantly reduced killing of GBS.

Because specific antibody is an important determinant in the opsonization, phagocytosis, and killing of GBS, we sought to investigate the role of PMN Fc receptors in the ingestion of opsonized GBS. Human PMN constitutively express two Fc receptors for IgG, FcRII and FcRIII, both of which bind multimeric IgG (23). FcRII is a transmembrane glycoprotein of 40 kDa recognized by MAb IV.3 and is present on the PMN surface at a density of 10,000 to 20,000 molecules per cell (21, 23, 28). On the other hand, FcRIII is expressed in neutrophils as a 50- to 80-kDa surface protein anchored to the cell membrane by a phosphatidylinositol glycan at 100,000 to 200,000 molecules per cell and is recognized by MAb 3G8 and Leu-11b (17, 22, 23). Tosi and Berger (28), Huizinga et al. (17), and Anderson et al. (1) have shown that FcRII and FcRIII of PMN have different roles in their interactions with complexed IgG. FcRIII, the more abundant of the two on the PMN cell surface, predominates in the binding of IgG-containing particles or complexes, whereas FcRII mediates activation of the respiratory burst and initiates IgG-induced phagocytosis. For this study, we used a radiolabeled uptake assay to measure the ingestion of opsonized GBS by PMN. We used MAb to the two PMN Fc receptors to assess the relative roles of these in the ingestion of opsonized GBS. Our findings indicate a major role for FcRII and an accessory role for FcRIII in the phagocytosis of GBS opsonized with IgG. By contrast, FcRII does not appear necessary for phagocytosis of GBS that have been opsonized with complement in addition to antibody, suggesting that the interaction of the complement-opsonized organism with complement receptors on PMN is sufficient to trigger phagocytosis.

MATERIALS AND METHODS

Cell preparation. Human PMN were isolated from peripheral blood of healthy adult volunteers as previously described (3). Briefly, fresh whole blood was obtained by venipuncture into a syringe containing citrate-dextrose phosphate (Abbott Laboratories, North Chicago, Ill.). PMN were purified by dextran sedimentation followed by centrifugation through a Ficoll (Sigma Chemical Co., St. Louis, Mo.)-

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Hypaque (Winthrop-Breon Laboratories, New York, N.Y.) gradient and hypotonic lysis of red blood cells. The purity and viability of PMN consistently exceeded 95%.

Bacteria. Strain M732 is a type III GBS isolated originally from the cerebrospinal fluid of an infant with purulent meningitis; its antibody and complement requirements for opsonophagocytosis and killing by PMN have been characterized previously (2, 13). M732 was grown to mid-log phase in glucose-enriched Todd-Hewitt broth containing [^{3}H]leucine, fixed with formalin, and lyophilized as described elsewhere (12). On the day of each experiment, an aliquot of the radiolabeled, lyophilized bacteria was suspended in Hanks balanced salt solution (HBSS) (GIBCO Laboratories, Grand Island, N.Y.) containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid), 1.5 mM $CaCl_2$, and 1.5 mM $MgCl_2$ ($HBSS^{++}$) at the appropriate dilution, and clumps were dispersed by gentle sonication.

Serum. Human immune serum was obtained from an adult volunteer known to have a moderate level of antibody to the capsular polysaccharide of type III GBS (9.0 μg/ml) by radioactive antigen-binding assay (5); approximately 68% of the antibody is IgG, 22% is IgA, and 10% is IgM, as determined by radioimmunoprecipitin assay (11). Whole blood was allowed to clot at room temperature, and the serum was separated by centrifugation at 4°C, aliquoted, and stored at −70°C until used. Hemolytic complement activity in the stored serum was well preserved. Complement inactivation of immune serum was achieved by heating the serum for 30 min at 56°C. For selected experiments, an IgG-rich fraction was obtained from immune serum by passage through a small QAE-Sephadex A-50 (Quick-Sep; Isolab Inc., Akron, Ohio) column followed by desalting and concentrating in a Centriprep-30 concentrator (Amicon, Danvers, Mass.). The specific antibody contents of the heated immune serum and the IgG-rich fraction were 8.5 and 6.8 μg/ml, respectively. The IgG-rich fraction, like the heated serum, had no hemolytic complement activity. Hypogammaglobulinemic serum was obtained from a patient with common variable immune deficiency, never treated with immunoglobulin, who had serum IgG and IgM concentrations of 48 and 30 mg/dl, respectively, and undetectable (<0.8 μg/ml) antibody to type III GBS. The bactericidal activities of the different sources of opsonins used were determined by an opsonophagocytic assay as described previously (10). Bacterial killing was expressed as the $\log_{10}$ of the reduction in colony counts after incubation for 40 min at 37°C of mid-log-phase M732 with PMN in the presence of the source of opsonin being tested.

Opsonization. Bacteria suspended in $HBSS^{++}$ were incubated at 37°C for 30 min with one of the opsonin sources mentioned above. After incubation, the opsonized bacteria were pelleted by centrifugation, washed twice with $HBSS^{++}$, and resuspended in $HBSS^{++}$ with 1% human serum albumin (Sigma) at a concentration equivalent to 5×10^8 CFU/ml. To verify the absence of residual complement opsonizing activity in heated immune serum and the IgG-rich fraction, C3 fragment deposition on M732 after incubation with the different opsonin sources was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) (8). This analysis revealed deposition of both C3b and iC3b on M732 when this strain was opsonized with intact immune serum or with hypogammaglobulinemic serum, but no C3 fragments were detected with heated immune serum or the IgG-rich fraction.

MAb. MAb IV.3 (IgG2b), which is directed to human FcRII (21), was purchased from Medarex, Inc. (West Lebanon, N.H.); the anti-FcRIII MAb Leu-11b (IgM) (22) was purchased from Becton Dickinson (San Jose, Calif.); and isotypic nonbinding controls MsIgM and MsIgG2b were purchased from Coulter Immunology (Hialeah, Fla.). Purified anti-HLA-A,B MAb W6/32 (IgG2b) (6), used as a control for binding to an irrelevant cell surface structure, was provided by Donald C. Anderson, Baylor College of Medicine, Houston, Tex. Leu-11b and IV.3 block the Fc-binding site of FcRIII and FcRII, respectively (21, 22). IV.3, Leu-11b, and W6/32 were found by flow cytometry to saturate PMN surfaces at concentrations of 5 to 10 μg/ml (25, 27). Control MAb were used at 5 μg/ml in the reaction mixture.

Phagocytosis. Phagocytosis of GBS was determined by measuring the uptake of opsonized, radiolabeled, formalin-fixed type III GBS by adult human PMN in suspension by a modification of the method of Verhoef et al. (29). Opsonized bacteria (10^8 CFU) were mixed with PMN (10^7 cells) in a total volume of 0.6 ml of $HBSS^{++}$ with 1% human serum albumin in the presence or absence of MAb. The mixture was rotated horizontally at 37°C for 20 min. As soon as the bacteria and PMN were mixed, duplicate 0.1-ml aliquots were removed and dissolved in Aquasol-2 (New England Nuclear Corp., Boston, Mass.) to determine the initial bacterial load in the reaction mixture. After 20 min of incubation, a second set of duplicate 0.1-ml aliquots was taken from the reaction mixture and diluted immediately in 1 ml of cold phosphate-buffered saline (PBS) containing 1 mM *N*-ethylmaleimide (Sigma) to stop phagocytosis. PMN were pelleted by centrifugation at $150 \times g$ for 4 min at 4°C. The pellet was then washed three times with the same solution and lysed with distilled water to release PMN-associated bacteria, which in turn were pelleted by centrifugation at $1{,}500 \times g$ for 30 min, and the resulting pellet was dissolved in Aquasol-2. The radioactivity of the initial and final aliquots was measured in duplicate in a liquid scintillation counter (model 2650 Tri-Carb liquid scintillation spectrometer; Packard Instrument Co., Inc., Laguna Hills, Calif.). Uptake was expressed as the percentage of radioactivity in the reaction mixture at zero time that was associated with the PMN pellet after 20 min of incubation. The uptake measured reflects both internalized and attached PMN-associated bacteria. The contribution of attached bacteria to total uptake was assessed by preventing internalization of attached particles (i.e., phagocytosis) by either incubating the reaction mixture at 0°C or incubating the reaction mixture in the presence of 1 mM *N*-ethylmaleimide at 37°C.

Blocking studies with MAb. Purified PMN were incubated for 15 min at room temperature in the presence of different concentrations of MAb. Without washing the cells, we then added bacteria to initiate phagocytosis and determined uptake as described above. Results are expressed as percent inhibition of uptake relative to values from identical tubes containing PBS diluent instead of MAb.

Statistical analysis. Data were analyzed by the Student unpaired *t* test (two tailed). The Bonferroni correction was applied when multiple comparisons were done (14).

RESULTS

Opsonin source. The uptake by normal adult PMN of opsonized M732 varied according to the opsonin source. Preliminary dose-response and kinetic experiments for each opsonin source revealed that maximal uptake of bacteria opsonized with hypogammaglobulinemic serum, intact immune serum, heated immune serum, and the IgG-rich frac-

TABLE 1. Uptake of opsonized GBS by human PMN

Opsonin source (concn)	Mean % uptake ± SEM (no. of expts)
Immune serum (25%)[a]	76.1 ± 2.1[b] (12)
Heated immune serum (40%)	21.7 ± 2.7 (18)
IgG-rich fraction (50%)	15.3 ± 0.9 (2)
Hypogammaglobulinemic serum (25%)[c]	29.1 ± 1.9[d] (5)
Heated immune serum (25%) + hypogammaglobulinemic serum (25%)	71.6 ± 2.1 (2)
$HBSS^{++}$	0.2 ± 0.1 (18)

[a] Type III GBS antibody content, 9.0 μg/ml.
[b] $P < 0.001$ compared with heated immune serum, IgG-rich fraction, or hypogammaglobulinemic serum.
[c] Undetectable antibody to type III GBS.
[d] $P < 0.017$ compared with heated immune serum or IgG-rich fraction.

tion of immune serum was achieved at concentrations of 25, 25, 40, and 50% (vol/vol), respectively, and that uptake reached a plateau between 15 and 30 min (data not shown). Opsonization with either antibody (heated immune serum or IgG-rich fraction) or complement (hypogammaglobulinemic serum) allowed moderate uptake by PMN (Table 1); this uptake was somewhat higher with complement than with antibody ($P < 0.017$). Consistent with previous observations (2), opsonization with immune serum containing both antibody and complement had a synergistic effect on uptake. Uptake rates with heated immune serum, the IgG-rich fraction, and hypogammaglobulinemic serum were 28, 20, and 38%, respectively, of the uptake occurring when intact immune serum was used as the opsonin source ($P < 0.001$, compared with intact immune serum). Heat inactivation of hypogammaglobulinemic serum abolished its opsonic activity (uptake, <0.5%). Opsonization with both heated immune serum and hypogammaglobulinemic serum resulted in uptake values comparable to those obtained with intact immune serum, confirming the synergy between antibody and complement. Uptake of nonopsonized bacteria consistently was less than 0.5%. As mentioned above, total uptake measured by this assay reflects both internalized and attached bacteria. To assess the portion of total uptake attributable to bacteria attached to the cell surface, we measured uptake under conditions that prevented phagocytosis. When the reaction mixture was incubated at 0°C or in the presence of 1 mM *N*-ethylmaleimide at 37°C, uptake values consistently were less than 20% of the uptake observed when phagocytosis was allowed.

When used as sources of opsonins in an assay of bactericidal activity, intact immune serum and hypogammaglobulinemic serum mediated reductions in the number of live M732 colonies by factors of >10 and $10^{0.2}$ to $10^{0.6}$, respectively. By contrast, heat-inactivated immune serum, the IgG-rich fraction of immune serum, and heat-inactivated hypogammaglobulinemic serum had negligible killing activity in the absence of a complement source.

Effect of MAb Leu-11b on GBS phagocytosis by PMN. To determine the role of the PMN FcRIII in the uptake of GBS, we studied the effect of blocking this receptor with MAb Leu-11b. Leu-11b produced a dose-dependent inhibition of uptake of GBS opsonized with intact immune serum or with heated immune serum, with a plateau reached at a MAb concentration of 5 to 10 μg/ml in the reaction mixture (Fig. 1). The degree of maximal inhibition for GBS opsonized with either intact immune serum or heat-inactivated immune serum was moderate (31% ± 3% and 40% ± 5%, respectively), without a significant difference between the two opsonin sources. Conversely, Leu-11b did not inhibit uptake of GBS opsonized with hypogammaglobulinemic serum. The nonbinding isotypic control MAb MsIgM and MsIgG2b caused no inhibition; uptake values were comparable to those of PBS controls. W6/32 caused a slight inhibition of uptake of GBS opsonized with heated immune serum (0 to 15%); this inhibition may have been due to interaction of the Fc portion of the bound MAb with adjacent Fc receptors (19). This Fc-FcR interaction is not possible with Leu-11b, since this MAb is of the IgM isotype.

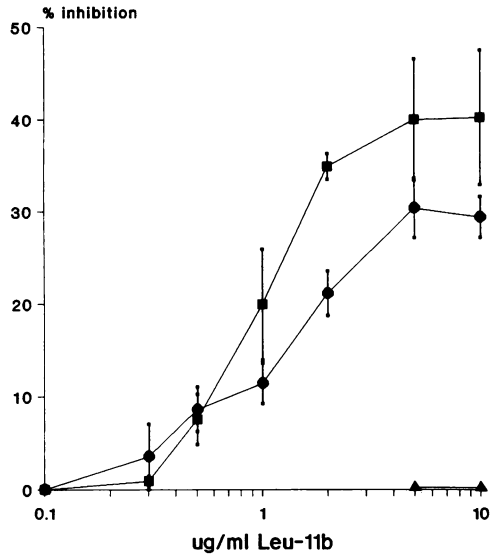

FIG. 1. Dose-dependent inhibition of opsonized GBS uptake by a MAb to FcRIII (Leu-11b). The M732 strain of GBS was opsonized with 25% immune serum (●), 40% heated immune serum (■), or 25% hypogammaglobulinemic serum (▲) as described in Materials and Methods and then incubated with PMN for 20 min at 37°C in the presence of Leu-11b at the indicated concentrations. Results are expressed as percent inhibition of GBS uptake by PMN relative to a control with PBS instead of MAb, and they represent mean values ± standard errors of the mean of four (●), eight (■), or two (▲) experiments.

Effect of MAb IV.3 on GBS phagocytosis by PMN. We studied the role of the PMN FcRII by blocking this receptor with MAb IV.3. The pattern of inhibition of uptake obtained by blocking FcRII was quite different from that obtained by blocking FcRIII. Figure 2 shows that IV.3 inhibited, in a dose-dependent fashion, the uptake of GBS opsonized with heated immune serum to a much greater magnitude ($P < 0.0001$) and at lower MAb concentrations than Leu-11b. A maximal inhibition of 77% was achieved with 0.5 μg of IV.3 per ml of reaction mixture. Surprisingly, inhibition of uptake of GBS opsonized with intact immune serum by IV.3 was consistently less than 4%, even with increasing MAb concentrations of up to 30 μg/ml ($P < 0.0001$). Like Leu-11b, IV.3 did not inhibit the uptake of GBS opsonized with hypogammaglobulinemic serum.

Effect of simultaneous blockade of FcRII and FcRIII with MAb. We also compared the inhibitory effects on the phagocytosis of opsonized GBS by both anti-Fc receptor MAb, alone and in combination, in the same experiment. Figure 3A shows an inhibition dose-response curve for GBS opsonized with heated immune serum. In a result similar to those in Fig. 1 and 2, increasing concentrations of Leu-11b or IV.3

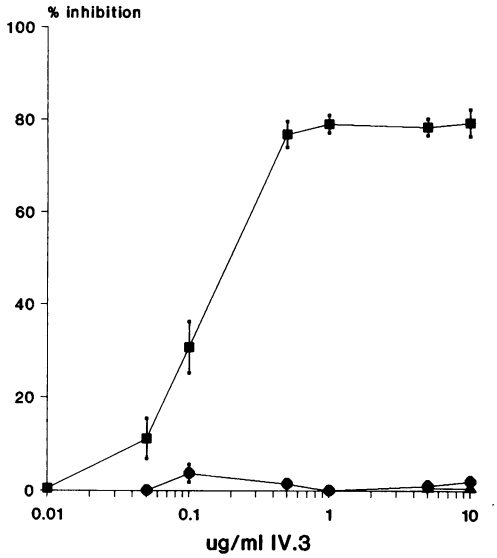

FIG. 2. Dose-dependent inhibition of opsonized GBS uptake by a MAb to FcRII (IV.3). The M732 strain of GBS was opsonized with 25% immune serum (●), 40% heated immune serum (■), or 25% hypogammaglobulinemic serum (▲) as described in Materials and Methods and then incubated with PMN for 20 min at 37°C in the presence of IV.3 at the indicated concentrations. Results are expressed as percent inhibition of GBS uptake by PMN relative to a control PBS instead of MAb, and they represent mean values ± standard errors of the mean of four (●), eight (■), or two (▲) experiments.

resulted in as much as 35% ± 4% or 75% ± 4% inhibition, respectively. When both MAb were used together, we observed an additive inhibitory effect on the uptake of GBS opsonized with heated immune serum to a maximum of 86% ± 1% ($P < 0.017$, compared with either MAb alone). By contrast, the inhibitory effect of Leu-11b alone on the uptake of GBS opsonized with intact immune serum was not augmented by the addition of IV.3 up to 10 μg/ml (Fig. 3B); similar results were obtained with GBS opsonized with both heated immune serum (source of antibody) and hypogammaglobulinemic serum (source of complement) simultaneously. When GBS was opsonized with the IgG-rich fraction of immune serum, patterns of uptake inhibition by MAb to Fc receptors mirrored those observed when GBS was opsonized with heated immune serum (Fig. 3C).

DISCUSSION

In this study, we investigated the participation of PMN Fc receptors in the phagocytosis of a type III GBS strain by assessing the inhibitory effect of receptor-specific MAb on the uptake of radiolabeled bacteria by PMN. Our findings indicate that FcRII plays a major role in mediating the ingestion of GBS opsonized only with antibody.

Opsonization of GBS with either antibody or complement allows moderate phagocytosis by PMN. Confirming what others have shown (2, 10, 13, 26), we found that phagocytosis is potentiated when both antibody and complement opsonize GBS. Contact between the GBS surface, coated with enough of either antibody or complement, and the PMN seems sufficient to trigger phagocytosis; however, the quantity of bacteria ingested is modest when only one kind of opsonin is present. Maximal ingestion takes place when both opsonins are present on the bacterial surface. Likewise, bacterial killing is maximal when both antibody and complement are present in sufficient amounts. By contrast, no bacterial killing can be demonstrated in the absence of complement, whether or not antibody is present. These findings indicate that the opsonic requirements for ingestion of type III GBS by PMN are not identical to those required for the intracellular killing of the bacteria.

A blockade of FcRIII inhibits only moderately the ingestion of GBS opsonized either with antibody alone or with antibody and complement. These findings suggest that the

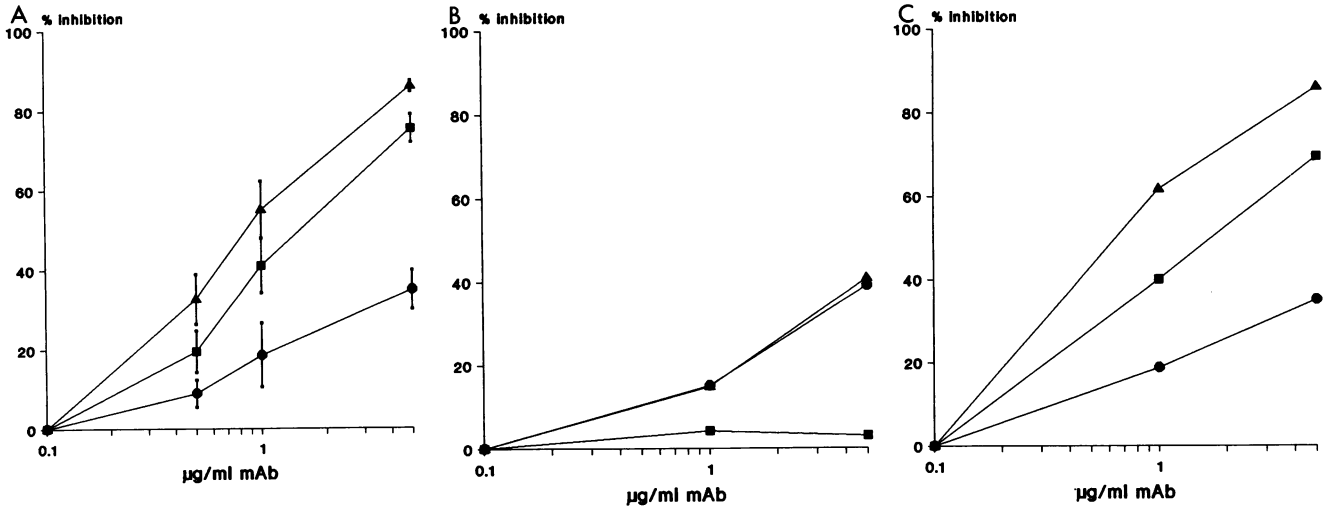

FIG. 3. Effect of simultaneous blockade of FcRII and FcRIII with MAb on uptake of opsonized GBS. M732 opsonized with heated immune serum (A), intact immune serum (B), or the IgG-rich fraction of immune serum (C) was incubated with PMN for 20 min at 37°C in the presence of the indicated concentrations of MAb Leu-11b (●), MAb IV.3 (■), or a combination of both MAb (▲) (actual IV.3 concentrations are 1/10 of what is shown on the x axis for both IV.3 alone and the combination). Results are expressed as percent inhibition of GBS uptake by PMN relative to a control with PBS instead of MAb, and they represent mean values ± standard errors of the mean of six experiments (A) or mean values of two experiments (B and C).

role of FcRIII in phagocytosis of GBS is in mediating attachment, perhaps anchoring bacteria on the PMN surface, thus facilitating its interaction with other PMN surface structures, as has been proposed by Tosi and Berger (28) and by Huizinga et al. (17). Binding of an anti-FcRIII MAb to the PMN cell surface could result in a partial blockade of FcRII through interaction between FcRII and Fc regions of bound anti-FcRIII. In the present study, however, we avoided such a possibility by using an anti-FcRIII MAb of the IgM isotype, Leu-11b. The reverse interaction, between FcRIII and Fc regions of bound anti-FcRII MAb, is of little consequence because FcRIII is much more abundant on the PMN surface than FcRII.

A blockade of FcRII strongly inhibited Fc-mediated phagocytosis of GBS. A simultaneous blockade of FcRIII augmented this high level of inhibition. Conversely, anti-FcRII MAb did not inhibit phagocytosis when GBS was opsonized with both antibody and complement. These findings indicate a primary role of FcRII in Fc-mediated phagocytosis of GBS. However, when the bacteria are also opsonized with complement, the need for FcRII is obviated.

Our findings are consistent with the concept that the primary role of FcRIII is of attachment of IgG-coated particles to the PMN surface, whereas the role of FcRII is that of IgG-mediated triggering of phagocytosis and the respiratory burst (1, 17, 28). The predominant role of FcRIII in binding IgG ligands is easy to understand because of the very large numbers of FcRIII molecules on the PMN surface. Because FcRII is a transmembrane protein, in contrast to FcRIII, which is anchored by a phosphatidylinositol glycan to the cell membrane outer leaflet, FcRII can effect intracellular signaling, whereas FcRIII may be unable to do so. This concept, however, may be too simplistic. There is mounting evidence that FcRIII may mediate some PMN responses. For example, lectin-mediated phagocytosis of concanavalin A-coated erythrocytes or of nonopsonized *Escherichia coli* is inhibited by MAb against FcRIII but not by MAb against FcRII (24). Similarly, ligation of FcRIII with MAb induces a rapid rise in intracellular calcium in PMN (18). Finally, FcRIII can mediate exocytosis of PMN granule proteins (16).

The importance of specific antibody in host defense against GBS is well recognized (4); however, our findings suggest that the role of Fc receptors in the ingestion by PMN of GBS opsonized with both antibody and complement is limited. Under these circumstances, FcRIII appears to play an accessory role similar to that which it plays when complement is not present, whereas FcRII, which predominates in Fc-mediated phagocytosis, appears not to be necessary for phagocytosis of GBS opsonized with both antibody and complement, since a blockade of FcRII with MAb was not inhibitory at all. We speculate that the interaction between opsonic complement fragments on the bacterial surface and PMN complement receptors provides the necessary signals to trigger and sustain phagocytosis. Perhaps the most important contribution of IgG to GBS ingestion, when complement is present, resides not in its direct interaction with PMN through Fc receptors but rather in its potentiation of the activation and deposition of complement on the bacterial surface. Antibody bound to the bacterial surface may increase the number of opsonic complement fragments deposited, promote deposition of complement at sites more accessible to phagocytes, including the antibody molecule itself, and protect bound complement fragments against degradation (7). What would then be the role of PMN Fc receptors in vivo during infection with GBS? PMN Fc receptors may mediate phagocytosis of antibody-opsonized GBS when complement is absent or present in very small amounts. In addition, Fc receptors appear to participate in the intracellular killing process after bacteria have been ingested (9, 20).

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