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Effect of Differences in Antibody and Complement Requirements on Phagocytic Uptake and Intracellular Killing of "c" Protein-Positive and -Negative Strains of Type II Group B Streptococci

NATHANIEL R. PAYNE,[1]† YOUNGKI KIM,[1] AND PATRICIA FERRIERI[1,2]*

Departments of Pediatrics[1] and Laboratory Medicine and Pathology,[2] University of Minnesota Hospital and Medical School, Minneapolis, Minnesota 55455

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The influence of antibody and complement on the polymorphonuclear leukocytic uptake and killing of type II group B streptococci (GBS) was examined with 11 adult sera and three type II strains possessing the trypsin-resistant and trypsin-sensitive components (II/TR+TS) of the "c" (formerly Ibc) protein or two type II strains lacking both components (II/no c) of the c protein. All tested sera mediated a >1 $\log_{10}$ reduction in colony-forming units (CFUR) of a type II/no c strain, even in the absence of measurable type-specific antibody (<1.08 μg/ml), but only 5 of 11 mediated a >1 $\log_{10}$ CFUR of any type II/TR+TS strain, even in the presence of moderate levels of type-specific antibody. The classical pathway of complement activation appeared to be more important than the alternative pathway, and even absorbed or immunoglobulin G (IgG)-depleted serum (IgG, 10 mg/dl) mediated a >1 $\log_{10}$ CFUR without magnesium ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (magnesium EGTA) chelation. Chelation with magnesium EGTA reduced the CFUR in 4 of 11 test sera and greatly reduced the CFUR in absorbed or IgG-depleted sera for type II/no c strains. Despite variation in the phagocytic killing of two representative strains of type II GBS, both strains were well phagocytized, as measured by radiolabeled bacterial uptake or electron microscopy. This study suggested that poorly killed type II/TR+TS GBS were easily phagocytized but apparently resisted intracellular killing.

Group B streptococci (GBS), important neonatal pathogens, are killed by phagocytes only after opsonization with complement and, in most cases, type-specific antibody (1, 4, 21, 24, 25). However, the interaction of GBS with polymorphonuclear leukocytes (PMN) and humoral immune factors may be more complex than originally thought and may vary among strains of the same serotype. Some clinical isolates of serotypes II and III appear to be more susceptible to opsonization and PMN killing than others (2, 7, 8, 21, 26), and some strains of serotype Ia GBS appear to be adequately opsonized by activation of the classical complement pathway alone, even in the absence of type-specific antibody (1). These in vitro differences in opsonic requirements among strains appear to correlate with differences in virulence in an animal model (6, 23).

In a previous study (21), differences in opsonic requirements among type II GBS clinical isolates correlated with the presence of both the trypsin-sensitive (TS) and trypsin-resistant (TR) components (II/TR+TS) of the "c" protein antigen (9, 11). However, the c protein antigen does not explain all the observed variations, and the presence of other protein antigens, such as the R proteins (18) or other, as-yet-unidentified factors, may be important.

This study was designed to (i) determine the relative importance of the classical and alternative complement pathways in the opsonization of type II GBS; (ii) correlate the concentration of antibody to type II capsular polysaccharide in serum with opsonizing efficiency; and (iii) determine whether some type II GBS strains were poorly killed because of inadequate opsonization and ingestion or poor intracellular killing after phagocytosis. The results of this study suggested that complement was primarily deposited on type II GBS by the classical complement pathway. However, opsonization with some magnesium ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (magnesium EGTA)-chelated sera also resulted in efficient PMN killing of selected type II GBS strains. There was no definite correlation between the concentration of type-specific antibody in serum and the promotion of PMN killing of poorly killed type II GBS strains. Both well-killed and poorly killed type II GBS strains were readily phagocytized, as demonstrated by radiolabeled bacterial uptake and electron microscopy. The observed differences in PMN killing of some type II GBS strains may be related to differences in the intracellular processing of ingested bacteria rather than resistance to phagocytosis.

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MATERIALS AND METHODS

Bacteria. Four type II/TR+TS GBS isolates, five type II/no c (lacking both components of the c protein complex) GBS isolates, and one nontypable clinical GBS isolate which had both the TS and TR components of the c protein antigen were processed, characterized, and stored as previously reported (11, 21). For bactericidal studies, 1-ml inocula were added to 3 ml of Todd-Hewitt broth, grown for 3 h at 35°C, washed twice, and suspended in phosphate-buffered saline (PBS) (pH 7.4) to an optical density at 620 nm which corresponded to 5×10^8 CFU/ml. Bacterial counts were confirmed by pour plating techniques. For radiolabeled bacterial uptake studies, 1 ml of stock culture was added to 10 ml of Todd-Hewitt broth containing 30 μCi of 2,8-^{3}H-

* Corresponding author.
† Present address: Department of Pediatrics, University of California at Los Angeles School of Medicine, Los Angeles, CA 90024.

labeled adenine, incubated for 3 to 18 h at 35°C, washed, and adjusted to 5×10^8 CFU/ml as noted above. The mean activity ± standard deviation per 5×10^6 bacteria was 7,763 ± 2,800 cpm.

Complement buffers. GVB++ contained 0.14 M NaCl, 0.01 M sodium barbital (Merck, & Co., Inc., Rahway, N.J.), 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 0.1% gelatin (Difco Laboratories, Detroit, Mich.) at pH 7.45. GVB-magnesium EGTA was the same as GVB++ but lacked $CaCl_2$ and contained 5 mM $MgCl_2$ and 10 mM EGTA (Sigma Chemical Co., St. Louis, Mo.). Inhibition of classical complement pathway activity by magnesium EGTA buffer was confirmed in a sheep erythrocyte (RBC) 50% hemolytic complement (CH_{50}) assay (21). Solutions used in the rabbit RBC CH_{50} assays were as previously published (20).

PMN. PMN were prepared as previously described (21) and suspended in Hanks balanced salt solution with 0.1% gelatin to 1×10^7/ml for the bactericidal assay and 5×10^6/ml for the radiolabeled bacterial uptake assay and electron microscopy. PMN suspensions contained 3 to 5% contaminating mononuclear cells and had ≥98% trypan blue exclusion at the conclusion of both the bactericidal and radiolabeled bacterial uptake assays.

Sera. Blood was donated by healthy adult volunteers after informed consent was obtained. All use of human subjects in this study received prior approval from the Committee on the Use of Human Subjects in Research of the University of Minnesota. Serum was processed and stored so as to preserve complement activity, as determined by sheep and rabbit RBC hemolysis assays. Antibody measurements were kindly performed by Carol Baker by a modified Farr technique (12). Selected sera were heat inactivated at 56°C for 60 min.

Absorbed sera. Selected sera were absorbed with a pelleted type II/no c GBS strain or a nontypable GBS strain bearing both components of the c protein complex. Bacteria were grown in Todd-Hewitt broth, washed twice in PBS, and the heated for 30 min at 60°C. Heat-killed bacteria were combined with the sera in a ratio of 1 volume of pelleted bacteria to 3 volumes of serum, and the mixture was rotated for 1 h at 4°C. Absorbed sera had concentrations of immunoglobulin G (IgG), IgM, C3, and C4 that were approximately 76 to 100% of preabsorption values and alternative and classical complement pathway activities that were 62 to 82% of preabsorption values. Immunoglobulins were measured by nephelometry on an Immunochemistry Systems Analyzer II (Beckman Instruments, Inc., Brea, Calif.), and C3 and C4 were measured by radial immunodiffusion (13).

C4 depletion of sera. Depletion of C4 in selected sera was accomplished by affinity chromatography. Briefly, 2 ml of goat anti-human C4 (Atlantic Antibodies, Scarborough, Maine) was coupled to 2 ml of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) in accordance with the instructions of the manufacturer. Spectrophotometric A_{280} measurements demonstrated greater than 99% binding of antibody to the Sepharose. A solution containing 0.5 M NaCl, 0.01 M sodium barbital, and 0.01 M EDTA was used as the column buffer, and regeneration of the column was accomplished with 3 M KSCN. Serum containing 0.01 M EDTA was passed over the column in 2-ml aliquots one to five times. The most concentrated fractions (determined by A_{280} measurements) were pooled and dialyzed against 1 liter of 0.5 M NaCl overnight, brought to 0.5 mM $MgCl_2$ with the addition of a concentrated $MgCl_2$ solution, and stored at −70°C until used. Serum depleted of C4 by this method contained 54 to 73% of the original C3 and no C4. Radial immunodiffusion was used to determine C3 and C4 concentrations with goat anti-human C3 and C4 (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) as previously published (13). The sheep RBC CH_{50} activity of the C4-depleted serum was undetectable (normal, 23 to 45 units). Alternative pathway activity was 47 to 54% of the pre-C4 depletion values, as determined by the rabbit RBC CH_{50} assay (20).

IgG depletion. Hypogammaglobulinemic serum was drawn from a patient with Bruton X-linked hypogammaglobulinemia just before administration of modified immune serum globulin. A cyanogen bromide-activated Sepharose 4B affinity column was prepared as described above for the C4 affinity column with 10 mg of protein A, which was greater than 99% bound to the Sepharose. Serum containing 0.01 M EDTA was passed over the column five times, pooled, and dialyzed against 0.5 M NaCl–0.01 M sodium barbital buffer. Before storage at −70°C, $CaCl_2$ and $MgCl_2$ were added to concentrations of 0.15 and 0.5 mM, respectively. After IgG depletion, this serum contained 58% of the initial sheep RBC CH_{50} activity, 53% of the original C4 and C3 concentrations, and IgG and IgM concentrations of 10 and 11 mg/dl, respectively.

Opsonization. Bacteria were opsonized in 10% untreated or heat-inactivated serum for 45 min at 35°C with end-over-end rotation at 10 rpm. The concentration of the C4-depleted or hypogammaglobulinemic serum used for opsonization was 20%, and that of the absorbed serum was 15%, to equalize complement activity, since absorbed and chromatographed sera became diluted during processing. Buffers used to dilute serum were GVB++ for untreated serum, PBS for heat-inactivated serum, and GVB-magnesium EGTA for chelated serum. Chelation was accomplished by combining serum and GVB-magnesium EGTA in a ratio of no less than 1:3 at room temperature for 5 min just prior to opsonization of bacteria.

Bactericidal assays. Bactericidal assays were performed as previously described (21), except that all bacteria were opsonized for 45 min at 37°C and washed once in PBS before addition to the reaction mixtures to prevent toxicity to the PMN when magnesium EGTA-chelated sera were used. The final reaction mixtures contained 5×10^6 PMN, 10^7 CFU of opsonized bacteria (bacterium/PMN ratio, 2:1), and Hanks balanced salt solution with 0.1% gelatin in a total volume of 1.0 ml. Reaction mixtures were sampled at zero time, incubated at 35°C with rotation at 10 rpm for 45 min, and sampled again. Bacteria incubated with serum alone or PMN alone did not show any reduction in CFU (CFUR). Positive controls for evaluation of different sera always included either serum A (type II-specific antibody, 29.75 μg/ml) or serum I (type II-specific antibody, 2.87 μg/ml). Positive controls for evaluation of different bacterial strains included either serum A or serum I and the easily killed type II/no c strain 79-176. Negative controls for evaluation of the easily killed type II/no c strain 79-176 always included heat-inactivated serum, which never resulted in >1 $\log_{10}$ CFUR. Calculation of CFUR was as follows: CFUR = ($\log_{10}$ CFU at zero time) − ($\log_{10}$ CFU at 45 min). Results represent the average of at least two experiments.

Radiolabeled bacterial uptake assays. Radiolabeled, 3-h cultures of selected GBS strains were washed and suspended to 5×10^7/ml. After opsonization, a 0.1-ml sample (5×10^6 CFU) was then counted and taken as the 100% control. Experimental samples contained 5×10^6 CFU and 5×10^5 PMN (bacterium/PMN ratio, 10:1) in a total volume of 0.2 ml of Hanks balanced salt solution with 0.1% gelatin in 4-ml

plastic vials (Bio-Rad Laboratories, Richmond, Calif.). For experiments in which bacterium/PMN ratios of 1:1 were used, 5×10^5 bacteria were added after culturing in 10 times the usual amount of radiolabeled adenine. Sample mixtures were incubated at 35°C on a platform shaker rotating at 150 rpm and were sampled at intervals from 0 to 90 min. After incubation, samples were washed twice with cold PBS and pelleted at 200 × *g* for 8 min. The remaining PMN pellet was suspended in scintillation cocktail (New England Nuclear Corp., Boston, Mass.) and counted in a model LS 150 scintillation counter (Beckman Instruments). Percent uptake was calculated as follows: sample cpm = [(PMN-associated bacteria)/(control cpm)] × 100, where control cpm represents 5×10^6 CFU alone. All experiments were repeated on at least one occasion. In the absence of PMN, there was a background uptake of 8 ± 3% for strain 79-176 and 11 ± 8% for strain 78-471.

Electron microscopy. Mixtures of PMN and bacteria for electron microscopy were prepared in a fashion identical to that used for radiolabeled bacterial uptake experiments, except that radioactive adenine was excluded from the growth medium. PMN and GBS were combined for 45 min at 35°C on a platform shaker rotating at 150 rpm, washed twice in ice-cold PBS, and pelleted at 200 × *g*. The pellet was processed for electron microscopy by fixation in 2.5% electron microscopy-grade glutaraldehyde (Ply Sciences, Fort Washington, Pa.) in Millonig buffer (1.7% monosodium phosphate, 0.36 NaOH, 0.54% glucose [pH 7.2]). PMN were then stained with 1.5% osmium tetraoxide (Lanum Co., Homewood, Ill.) in Millonig buffer for 1 h. Following dehydration in progressively more-concentrated ethanol solutions, specimens were embedded in Polybed (Polysciences, Warrington, Pa.), sectioned, and examined with a Philips 201 electron microscope (Philips, Eindhoven, The Netherlands) at 5,140 to 40,000×. Quantitation of the number of bacteria inside PMN was done by examining photographs of an average of 53 PMN from each sample mixture photographed at a magnification of 5,140×. Only those bacteria clearly within a phagocytic vacuole were recorded as being intracellular. Bacteria were counted as attached if they were partially enveloped by but not surrounded by the PMN membrane. From these observations, the average number of ingested cocci and the total number of cell-associated (attached plus ingested) cocci per PMN were derived. Only PMN whose nucleus and entire cytoplasm appeared in the photographs were counted.

Immunofluorescence staining. Bacterial suspensions were prepared and opsonized as described for the bactericidal assay. Bacteria were air dried on glass slides and stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgM, IgA, and C3, rabbit anti-human IgG (Cooper Biomedical, Inc., West Chester, Pa.), mouse monoclonal anti-human poly-C9 (5), or sheep anti-fibronectin (Miles Scientific). Slides were processed and stained as previously described (10) with the selected FITC-conjugated antibody on a single day to minimize variations in lots of antibody and in processing techniques. Unopsonized bacterial controls were included for each antibody and were routinely negative. Stained slides were examined by two observers (N.R.P. and P.F.) independently with a Seitz Universal microscope and given a grade of 0 to 3+ fluorescence without a knowledge of the identity of the slides. The subjective grade of fluorescence was assigned a numerical value (0 = 0, trace = 0.5, 1+ = 1, 2+ = 2, and 3+ = 3), and the two observers' scores were averaged to derive a final score for each serum-GBS strain combination. All final scores were averaged to calculate a mean score for each strain. Strains were then compared to determine relative differences in the deposition of IgG, IgM, IgA, C3, and fibronectin on the test strains.

TABLE 1. Log_{10} CFUR at 45 min in a bactericidal assay with adult volunteer sera

Donor[a]	Concn (μg/ml) of type II-specific antibody[b]	Log_{10} CFUR of indicated strain:				
		Type II/no c		Type II/TR+TS		
		79-176	74-692	78-471	80-103	77-532
D	35.92	1.30	1.05	0.98	1.10	0.83
A	29.75	1.35	0.90	1.24	1.20	1.35
H	26.57	1.58	0.99	0.68	1.01	0.81
G	10.75	1.10	0.92	0.57	0.81	0.89
F	8.26	1.45	0.80	0.68	0.83	1.48
J	3.22	1.58	0.99	0.60	0.21	0.55
I	2.87	1.43	0.73	0.83	1.39	0.57
C	2.38	1.39	0.79	0.24	0.44	0.34
E	1.40	1.58	0.99	0.51	0.04	0.54
B	<1.08	1.59	0.81	0.26	0.50	0.22
K	<1.08	1.51	0.95	0.54	0.90	0.37
I-abs[c]	<1.08	1.22				
L[d]	ND	1.02				

[a] Donors are listed in descending order of antibody concentration.
[b] Measured by Carol Baker, Baylor College of Medicine. ND, Not done.
[c] Absorbed with strain 79-176.
[d] IgG-depleted serum (total IgG, 10 mg/dl; total IgM, 11 mg/dl).

Statistical methods. Results of bactericidal assays were analyzed by unpaired *t* tests. Radiolabeled bacterial uptake results were evaluated by analysis of variance and Student's *t* test. Correlation coefficient calculations were used to compare the CFUR results with the concentration of type II-specific antibody in test sera. A value of $P \leq 0.05$ was considered statistically significant.

RESULTS

Strain variations. Type II/TR+TS and type II/no c GBS strains were opsonized with sera from 11 adult human volunteers. Significant strain variations among serotype II GBS in susceptibility to PMN killing were seen. The presence of both the TR and TS components of the c protein was associated with resistance to PMN killing in the bactericidal assay. Only 5 sera were able to mediate a ≥1 log_{10} CFUR of any of the type II/TR+TS strains, and only 1 of 11 sera was associated with a 1 log_{10} CFUR of all three type II/TR+TS strains (Table 1). The three type II/TR+TS strains had an average log_{10} CFUR at 45 min of 0.71 ± 0.36 (mean ± standard deviation) when tested against the 11 adult sera, as compared with 1.15 ± 0.35 for the two type II/no c strains (Table 1) ($P < 0.001$, unpaired *t* test). However, the presence of the c antigen did not fully explain all strain variations. Opsonization with 11 adult sera resulted in a 1.44 ± 0.24 log_{10} CFUR of strain 79-176, a type II/no c strain, but only a 0.86 ± 0.15 log_{10} CFUR of strain 74-692, also a type II/no c strain ($P < 0.001$, unpaired *t* test).

Concentrations of anti-GBS antibody in serum and opsonic activity. No clear relationship emerged between the concentrations of type II-specific antibody in test sera and the ability to mediate phagocytic killing of any serotype II strain tested. All 11 sera tested mediated a ≥1 log_{10} CFUR of strain 79-176, a type II/no c strain, despite undetectable levels (<1.08 μg/ml) of antibody to type II capsular polysaccharide in 2 of 11 sera (Table 1).

Opsonization of type II/TR+TS strains also did not correlate with type II-specific antibody levels (correlation coef-

TABLE 2. Log_{10} CFUR of strain 79-176 at 45 min in a bactericidal assay with fresh, chelated, and C4-depleted sera

Serum	Log_{10} CFUR (mean ± SD) with serum:	
	I[a]	B[b]
Fresh, untreated	1.38 ± 0.20	1.98 ± 0.44
Magnesium EGTA-chelated	2.17 ± 0.20	2.34 ± 0.13
C4-depleted	1.83 ± 0.35	1.87 ± 0.26

[a] Type II-specific antibody concentration, 2.87 μg/ml.
[b] Type II-specific antibody concentration, <1.08 μg/ml.

ficient, −0.484). Despite antibody levels as high as 26.57 μg/ml, serum H mediated only a 0.68 to 0.81 log_{10} CFUR of two of the three type II/TR+TS strains (Table 1). Only five sera supported opsonization of any type II/TR+TS strain and resulted in a ≥1 log_{10} CFUR. Those sera varied in concentrations of type II-specific antibody from 2.87 to 35.92 μg/ml.

Four sera demonstrating poor opsonic activity for the type II/TR+TS GBS strain 78-471 were used in concentrations of 20 to 50% to see if increasing the serum concentration would influence PMN phagocytic killing of type II GBS. With one of the four sera (type II-specific antibody level, 1.40 μg/ml), the CFUR increased from 0.82 log_{10} at 10% serum to 1.56 log_{10} at 50% serum.

When three other sera (range of type II-specific antibody levels, <1.08 to 3.22 μg/ml) were tested at 40 or 50% concentrations, the number of bacteria killed differed from the CFUR at 10% serum by ≤ 0.15 log_{10} and did not exceed 1 log_{10} CFUR in any instance.

Absorbed and hypogammaglobulinemic sera. Two sera (sera I and B, type II-specific antibody levels, 2.87 and <1.08 μg/ml, respectively) were absorbed with either strain 79-176, a type II/no c GBS strain (homologous absorption), or strain 80-042, a nontypable GBS isolate bearing both TR and TS components of the c protein antigen but no identifiable type polysaccharide (heterologous absorption). Opsonization of strain 79-176 with serum B after homologous absorption resulted in a 1.17 log_{10} CFUR; opsonization of strain 80-042 after heterologous absorption resulted in a 1.33 log_{10} CFUR, as compared with a 1.44 log_{10} CFUR before absorption. Similarly, opsonization with serum I after homologous absorption with strain 79-176 or heterologous absorption with strain 80-042 altered by CFUR by a maximum of 0.16 log_{10}, in the absence of magnesium EGTA chelation. The absorption technique was effective in removing type II-specific antibody and resulted in a decrease from 2.87 μg/ml to an undetectable level (<1.08 μg/ml). Chelation of absorbed serum B with magnesium EGTA altered the CFUR from 1.17 to 0.23 log_{10}.

Similar results were seen in the bactericidal assay after opsonization of strain 79-176 with IgG-depleted serum. The IgG-depleted serum (IgG, 10 mg/dl; IgM, 11 mg/dl) mediated a CFUR of 1.02 ± 0.31 log_{10} before chelation but a CFUR of only 0.56 ± 0.27 log_{10} after magnesium EGTA chelation. After absorption or in the presence of extremely low levels of immunoglobulin, the classical pathway appears to be adequately activated.

Chelation of sera. The ability of type II GBS to activate complement by the classical or alternative pathway or both was determined by comparing magnesium EGTA-chelated sera with fresh, untreated sera. Opsonization of strain 79-176 with 7 of 11 sera after chelation resulted in either no change in the CFUR or a slight increase as compared with the results obtained with unchelated sera (means, 1.46 ± 0.51 versus 1.44 ± 0.24 log_{10}, respectively). However, 4 of 11 test sera were less effective in opsonizing and killing this strain after chelation and mediated a CFUR 0.41 to 0.76 log_{10} units lower than that mediated by unchelated sera. There was no relationship between type II-specific antibody concentrations and CFUR after opsonization with chelated sera. Opsonization of strain 78-471 with chelated sera containing <1.08 or 2.87 μg of type II-specific antibody (donors B, I, and K) per ml resulted in a CFUR either the same as or up to 16% greater than that obtained with fresh, untreated sera. To ascertain that magnesium EGTA chelation effectively inhibited classical complement pathway activity, we depleted sera I and B of C4 and then tested them for the ability to support phagocytic killing of strain 79-176. In each case, C4-depleted sera mediated a CFUR very similar to that mediated by magnesium EGTA-chelated sera and equal to or greater than that mediated by fresh, untreated sera (Table 2). Thus, both C4-depleted and magnesium EGTA-chelated sera supported opsonization, suggesting that, for some sera, the alternative pathway of complement activation, in addition to the classical pathway, may be important in the opsonization of type II GBS.

Radiolabeled bacterial uptake. Since there were significant differences between the killing of type II/TR+TS and type II/no c GBS strains, it was necessary to identify whether those differences were due to decreased phagocytosis or to decreased intracellular killing of ingested bacteria. Radiolabeled bacterial uptake assays were designed to measure phagocytosis. Type II strains were rapidly taken up by PMN: one-third to one-half of the total radiolabeled bacterial uptake occurred after 1 min, was almost maximal after 15 min, and reached a plateau by 30 min (Fig. 1). Extending the length of bacterial culturing from 3 to 18 h (data not shown) or using a bacterium/PMN ratio of 1:1 (Fig. 1) yielded similar results. Despite marked differences among the type II GBS strains in the bactericidal assays, few differences emerged in the radiolabeled bacterial uptake experiments. For example, the radiolabeled bacterial uptake after opsonization with serum B of a representative type II/no c strain was very similar to that of a type II/TR+TS strain (44 versus 50%;

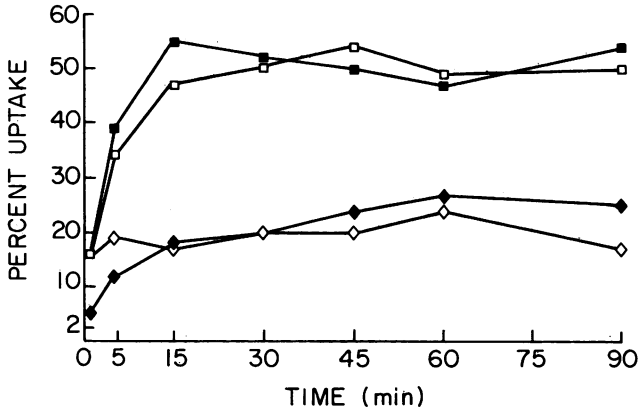


FIG. 1. Radiolabeled bacterial uptake. PMN uptake of opsonized and unopsonized radiolabeled GBS was rapid and reached a plateau after 15 min for all strains tested. There were no differences in the amount of bacterial uptake between a 1:1 and a 10:1 bacterium/PMN ratio. Symbols: ◆, unopsonized type II/no c strain (bacterium/PMN ratio, 1:1); ◇, unopsonized type II/no c strain (bacterium/PMN ratio, 10:1); □, type II/no c GBS strain opsonized with serum I (bacterium/PMN ratio, 10:1); ■, type II/no c GBS strain opsonized with serum I (bacterium/PMN ratio, 1:1).

TABLE 3. Mean percent PMN uptake of radiolabeled type II GBS after opsonization with untreated and treated sera

Strain and serum	% Uptake with indicated serum (bacterium/PMN ratio):					
	Untreated (10:1)	Chelated (10:1)	C4 depleted (10:1)	Absorbed[a] (10:1)	Heated[b] (10:1)	Untreated (1:1)
Type II/TR+TS (78-471)						
I	54	51	52	57	45	67
B	50	42	44	41	44	50
None	41					
Type II/no c (79-176)						
I	54	53	55	44	51	50
B	44	49	49	36	40	55
None	20					

[a] Absorbed with the same strain examined.
[b] Heated for 1 h at 56°C.

Table 3), despite marked differences in phagocytic killing (1.59 versus 0.26 $\log_{10}$ CFUR; Table 1). In contrast to the results of the bactericidal assays, opsonization of type II GBS with heat-inactivated or untreated serum B resulted in small differences in radiolabeled bacterial uptake. For example, strain 79-176, a type II/no c strain, had 44% uptake when opsonized with untreated serum B, 40% uptake when opsonized with chelated serum B, 49% uptake when opsonized with C4-depleted serum B, 36% uptake when opsonized with absorbed serum B, and 40% uptake when opsonized with heat-inactivated serum B ($P = 0.052$, analysis of variance). Opsonization with absorbed sera frequently resulted in a slightly lower uptake than did opsonization with other treated sera (Table 3). Similar results were seen with strain 79-176 opsonized with untreated, chelated, C4-depleted, absorbed, or heat-inactivated serum I ($P = 0.229$, analysis of variance). Strain 78-471, a type II/TR+TS strain, also had equivalent uptake with sera I and B after C4 depletion, chelation, absorption, heat inactivation, or no treatment.

Bacterial killing was never observed in the bactericidal assay in the absence of serum. However, in PBS alone, PMN uptake of strain 78-471 occurred regularly (41 ± 2%), and uptake of strain 79-176 also occurred to some extent (20 ± 2%) (Table 3). These findings were noted consistently (n = 12) and suggested attachment to or ingestion by PMN or both in the absence of serum (Fig. 2A). Three additional type II/TR+TS strains were also examined in the unopsonized state, yielding similar results (data not shown). PMN uptake of GBS in the radiolabeled bacterial uptake assay was confirmed by light microscopic examination of PMN and GBS mixtures after Wright staining and on separate occasions after Gram staining of slides.

Electron microscopy. To confirm that radiolabeled bacterial uptake correlated with phagocytosis, we examined uptake assay specimens by electron microscopy at a magnification of 5,140×. Bacteria were counted as ingested if they were completely enclosed within a phagocytic vacuole (Fig. 2A). If GBS were partially engulfed by phagocytic pseudopods but not internalized, they were counted as attached (Fig. 2A). Bacteria touching PMN but not partially surrounded by pseudopods were rare and were not counted as PMN associated.

Few PMN-associated bacteria were not internalized; unopsonized strain 78-471 had the most partially engulfed but not ingested GBS, 0.6 cocci per PMN or 28% of the total PMN-associated bacteria (Table 4 and Fig. 2A). GBS attachment to the PMN surface was also seen with strain 78-471 after opsonization with sera that had been heat inactivated, chelated, absorbed, or depleted of C4 (0.1 to 0.2 cocci per PMN). After oponization of strain 78-471 with untreated serum, 3.2 to 3.6 cocci per PMN were internalized, and after opsonization with heat-inactivated serum, 2.2 to 2.6 cocci per PMN were identified within phagocytic vacuoles (Table 4 and Fig. 2B).

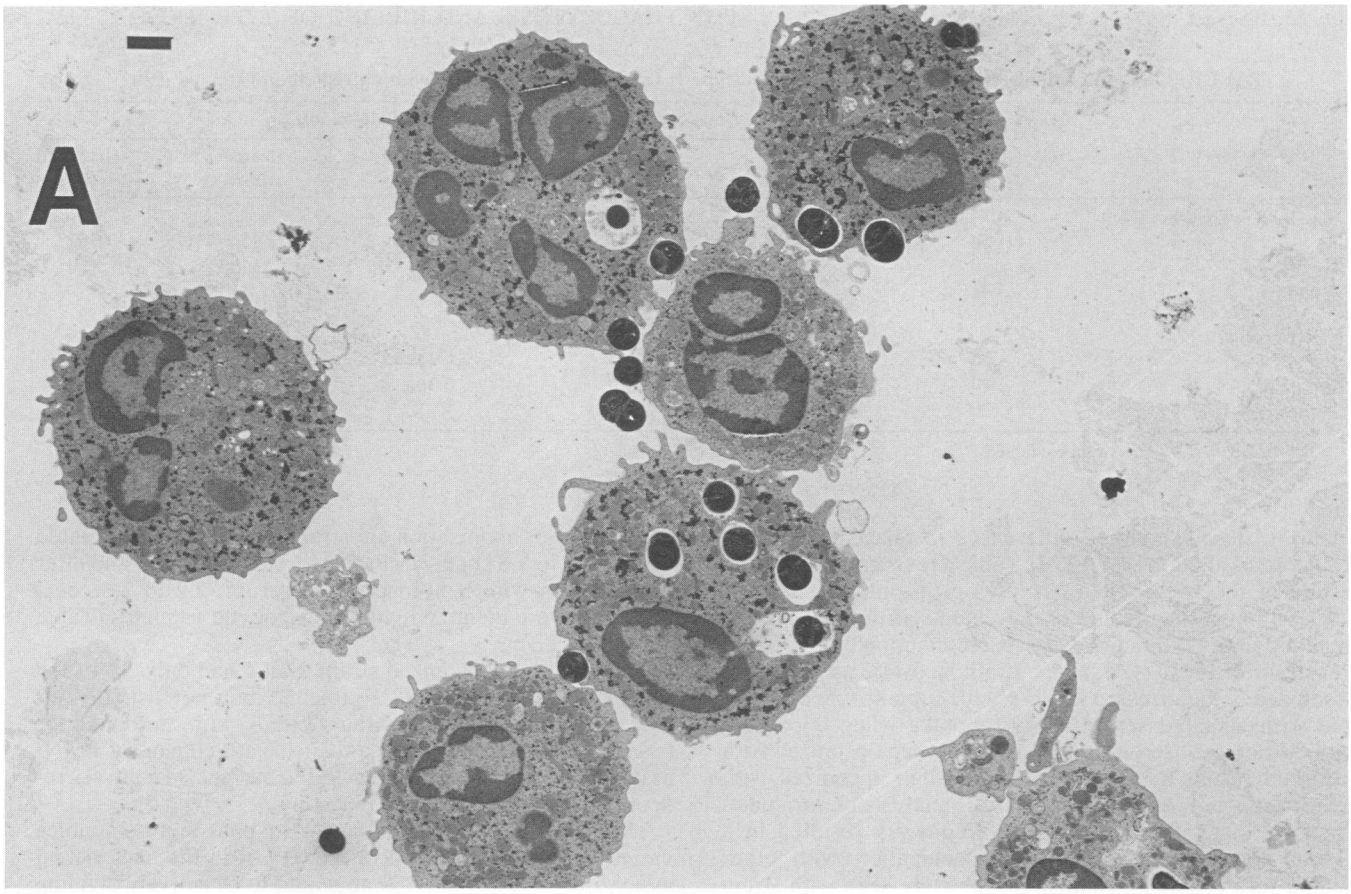
In contrast, unopsonized strain 79-176 had only 3% of the total PMN-associated GBS attached but not actually ingested. Opsonization of strain 79-176 with untreated or treated serum resulted in less than 1% attachment of PMN-associated GBS to the outer PMN surface (Table 4), reflecting efficient phagocytic ingestion.

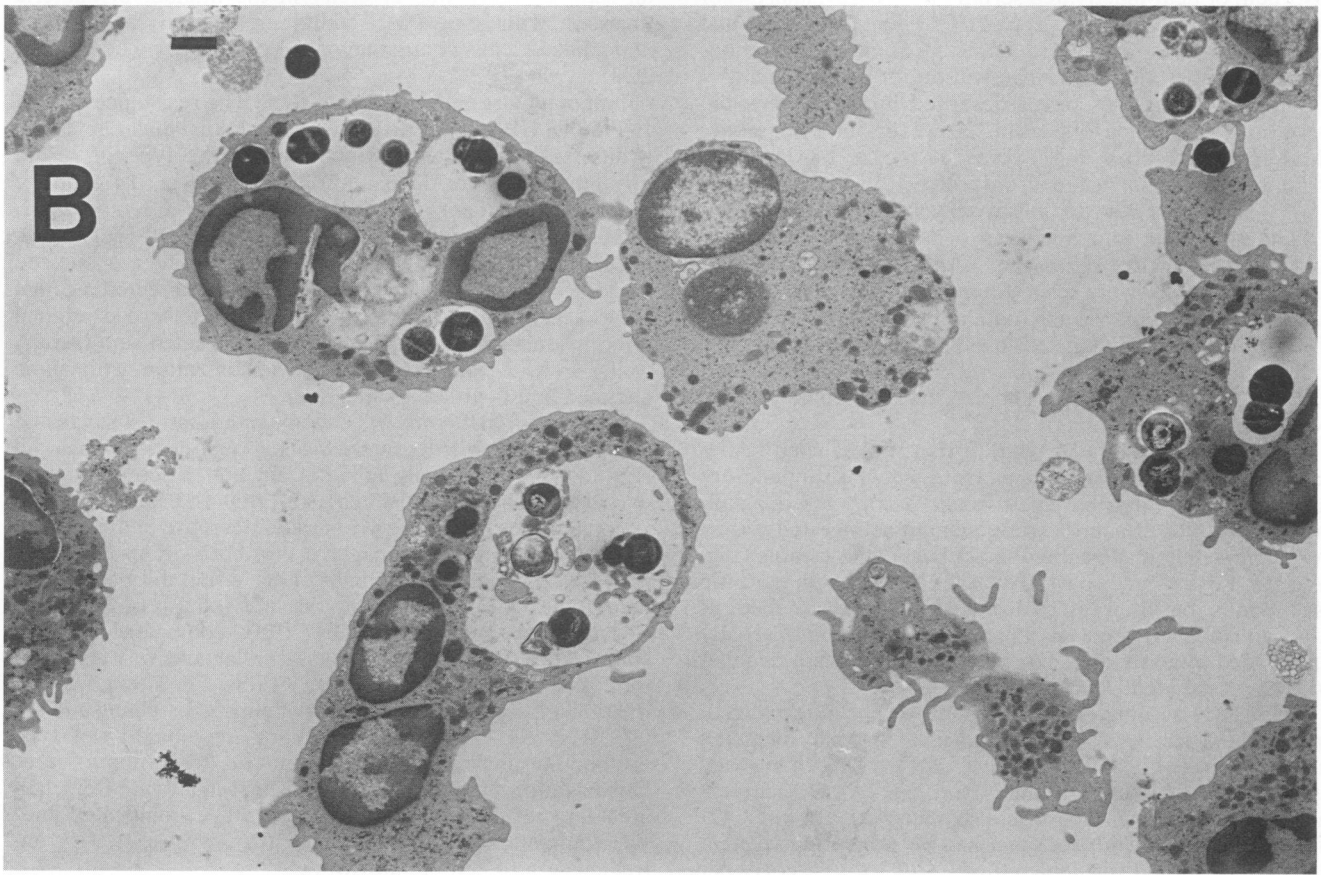
The appearance of bacteria within phagocytic vacuoles varied and was not related to the CFUR in the bactericidal assay. Bacteria within PMN appeared to be actively dividing in some cases and in others to be undergoing degradation. In all samples there was diversity in the number of bacteria ingested, with some PMN containing as many as 14 cocci and adjacent cells containing only 1 coccus or no cocci (Fig. 2C).

Immunofluorescence studies of GBS. The resistance of type II/TR+TS GBS strains to intracellular killing might be due to a difference in either the quantity or the type of serum opsonins that were deposited on the bacteria. To estimate the relative amounts of serum components C3, IgG, IgM, IgA, and fibronectin on a type II/no c strain (79-176) and on a type II/TR+TS strain (78-471), we stained opsonized bacteria with FITC-conjugated antibody directed against these serum opsonins. These two strains were examined after opsonization with nine different untreated sera (sera A to D, F to I, and K) and after opsonization with some chelated and heat-inactivated sera.

Staining of GBS with FITC-conjugated anti-C3 suggested that the poorly killed strain, 78-471, bound less C3 than did strain 79-176, the easily killed strain (mean scores ± standard deviations, 1.3 ± 0.4 versus 2.1 ± 0.4). Chelation of the opsonizing sera resulted in a marked decrease in the anti-C3 staining to barely detectable levels for both strains (Table 5). Anti-C3 staining and CFUR of either strain did not show a consistent relationship among the individual sera.

Fluorescence of bacteria after FITC-conjugated anti-IgG staining of these two strains was similar and was less than that seen with anti-C3 staining (Table 5). There was no relationship between the amount of anti-IgG staining and the CFUR in the bactericidal assay or the quantity of type II-specific antibody present in the opsonizing sera. Opsonization of both strains with heat-inactivated or chelated sera resulted in slightly greater FITC-conjugated anti-IgG immunofluorescence staining than was seen with untreated sera (data not shown).


A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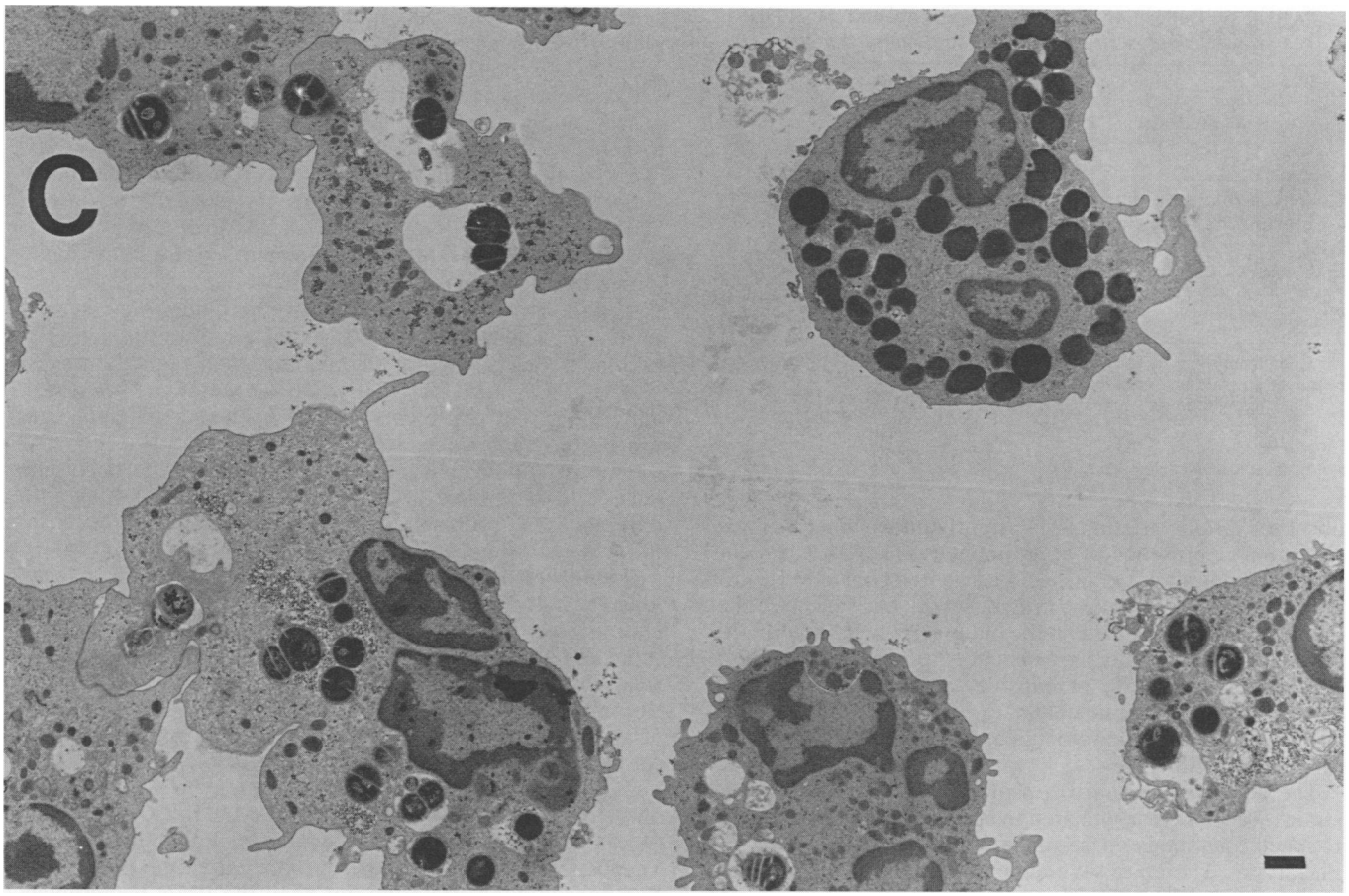

FIG. 2. (A) Type II/TR+TS strain 78-471 after incubation with PMN for 45 min in serum-free buffer. The CFUR for this mixture in the bactericidal assay was 0.06 $\log_{10}$ units, and the radiolabeled bacterial uptake was 41% of that in the control. Magnification, ×5,140; bar, 1 μm. (B) Type II/TR+TS strain 78-471 after incubation with PMN for 45 min after preopsonization with 10% serum B (type II-specific antibody level, <1.08 μg/ml). The CFUR for this mixture in the bactericidal assay was 0.09 $\log_{10}$ units, and the radiolabeled bacterial uptake was 44%. Magnification and bar are as in panel A. (C) Type II/no c strain 79-176 phagocytized by PMN during 45 min of incubation after preopsonization with 10% serum I (type II-specific antibody level, 2.87 μg/ml). Some PMN have ingested many GBS, while others have only one or no GBS internalized. In the bactericidal assay a 1.43 $\log_{10}$ CFUR was recorded for this strain and serum. The radiolabeled bacterial uptake was 54%. Magnification and bar are in panel A.

TABLE 4. Comparison of PMN killing, radiolabeled bacterial uptake, and ingestion and attachment of type II GBS

Strain and serum	$\log_{10}$ CFUR (mean ± SEM)	% uptake (mean ± SEM)	Avg no. of cocci/cell	
			Ingested	Attached but uningested
Type II/TR+TS (78-471)				
None	0.06	41 ± 2	1.4	0.6
I	0.83 ± 0.16	54 ± 2	3.2	0.2
Heated I	0.39 ± 0.09	45 ± 2	2.6	0.1
B	0.26 ± 0.05	50 ± 3	3.6	0.2
Heated B	0.09 ± 0.02	44 ± 4	2.2	0.2
Type II/no c (79-176)				
None	0.06[a]	20 ± 2	1.5	0.1
I	1.43 ± 0.10	54 ± 2	4.1	0.0
Heated I	0.49 ± 0.11	51 ± 2	3.5	0.0
B	1.59 ± 0.17	44 ± 3	3.4	0.0
Heated B	0.11 ± 0.04	40	3.5	0.0

[a] CFU increase, not decrease.

Strain 78-471 stained more intensely with FITC-labeled anti-IgA and anti-IgM than did strain 79-176 (Table 5). For example, the mean scores with anti-IgA were 1.5 ± 0.6 for the type II/TR+TS strain and 0.1 ± 0.1 for the type II/no c strain.

Fluorescence after FITC-conjugated anti-fibronectin antibody staining was modest for both strains, the mean scores being 0.5 ± 0.3 for the type II/no c strain and 1.0 ± 0.6 for the type II/TR+TS strain. The amount of fluorescence varied little regardless of which serum was used for opsonization. No poly-C9 was detected on either of these two GBS strains.

DISCUSSION

Variation in the opsonization and phagocytic killing requirements of type II GBS has been reported previously (2, 21) and was again observed in this study. The presence of both the TS and TR components of the c protein antigen partially explains some type II GBS strain resistance to phagocytic killing by PMN (21). There are certainly other determinants as well, since strains lacking the c protein may also demonstrate strain variation. The other sources of the

TABLE 5. Deposition of serum components on type II GBS strains as assessed by immunofluorescence staining

Serum component	Mean visual score[a] of degree of fluorescence of strain:	
	79-176 (type II/no c)	78-471 (type II/TR+TS)
C3 (opsonizing sera, untreated)	2.1 ± 0.4	1.3 ± 0.4
C3 (opsonizing sera, chelated)[b]	0.8 ± 0.3[c]	0.2 ± 0.1[d]
IgG[e]	0.8 ± 0.4	0.9 ± 0.6
IgM[e]	0.5 ± 0.2	1.6 ± 1.5
IgA[e]	0.1 ± 0.1	1.5 ± 0.6
Fibronectin[e]	0.5 ± 0.3	1.0 ± 0.6

[a] Composite score of the amount of fluorescence with the opsonizing sera examined (n = 9, unless otherwise indicated).
[b] Chelated with magnesium EGTA before opsonization.
[c] $n = 8$.
[d] $n = 3$.
[e] Untreated opsonizing sera.

observed strain variation are currently unexplained but may relate to the presence of other antigens, such as R proteins (18), the amount of cell-associated lipoteichoic acid (19), or as-yet-unidentified factors. Previous work has suggested that the quantity of sialic acid on the outer surface of type II GBS does not play a role in resistance to opsonization (26). Although the quantity of capsular polysaccharide antigen has been associated with enhanced virulence of type III GBS strains in a chicken embryo model (14), no information exists for type II GBS strains.

The ability of serum to opsonize different type II GBS strains was not clearly related to concentrations of total type II-specific antibody in serum, as measured by a modified Farr assay. There are several potential reasons for this lack of correlation. First, besides type II-specific polysaccharide there are probably other antigens to which opsonically active antibody may be directed. Examples of such antigens are bacterial surface protein antigens such as components of the c proteins (11, 15), R proteins (18), other unidentified proteins, or polysaccharide moieties of the bacterial cell wall other than type II-specific polysaccharide. Antibodies directed against these antigens would not be measured in an antibody assay with purified type II-specific polysaccharide.

The second possible explanation for a poor correlation between type II-specific antibody concentrations and opsonic activity is the inhibition of type II-specific IgG deposition on the type II GBS by some component(s) of the bacterial surface. Russell-Jones et al. (22) have shown that the c protein can bind IgA. The results of the present study are consistent with the binding of IgA by the c protein. There was increased fluorescence after FITC-conjugated anti-IgA staining of opsonized type II/TR+TS strain 78-471 as compared with type II/no c strain 79-176. The c protein antigen appears to be located on the surface of the bacterial cell wall (27), and IgA deposited on the c protein could conceivably block the deposition of opsonically effective IgG or complement.

The importance of the complement system is underscored by the finding that IgG-depleted (IgG, 10 mg/dl; IgM, 11 mg/dl) serum could mediate a >1 $\log_{10}$ CFUR. Furthermore, unchelated serum containing low levels of type II-specific antibody when absorbed with type II/no c GBS (antibody undetectable after absorption) also supported a >1 $\log_{10}$ CFUR. However, magnesium EGTA chelation of the hypogammaglobulinemic or absorbed serum markedly decreased phagocytic killing. These results suggest that classical complement pathway activation may occur in the absence of type II-specific antibody and that alternative complement pathway activation is not clearly linked to the quantity of type II-specific antibody.

Complement activation by GBS in the absence of type-specific antibody has been reported previously for type Ia (1) and II (2) GBS and also for type Ia (16)- and possibly type III (17)-specific polysaccharides. The mechanism for this interaction is not known but appears to involve the direct activation of C1 by the GBS polysaccharides. The role that this direct activation of the complement system might have in neonatal infections is not known.

Variations in complement activity may determine serum opsonizing ability. However, examination of this hypothesis by Baker et al. (2) did not identify any significant differences in CH_{50}, C3, C4, factor B antigen, factor B functional activity, or control proteins H and I when sera with high and low bactericidal indices were compared.

The variation in phagocytic killing among type II strains led us to determine whether this variation was due to differences in opsonization and ingestion or to differences in intracellular killing after successful phagocytosis by PMN.

Radiolabeled bacterial uptake suggested that the poorly killed type II/TR+TS strains and the easily killed type II/no c strains were both phagocytized readily. Radiolabeled bacterial uptake of type II/TR+TS strain 78-471 was greater than that of type II/no c strain 79-176 after both were opsonized with serum B. In contrast, the CFUR of the two strains showed that even though it showed greater uptake, the type II/TR+TS strain had only a 0.26 $\log_{10}$ CFUR, as compared with a 1.59 $\log_{10}$ CFUR for the type II/no c strain. Electron microscopy confirmed that type II/TR+TS strain 78-471 did enter PMN and was contained within intracellular vacuoles (Fig. 2B and C). Therefore, after internalization this poorly killed strain did not elicit the PMN bactericidal response, suppressed the response, or was somehow resistant to the PMN efforts to destroy the internalized bacteria.

Earlier work (21) from this laboratory suggested that the poorly killed type II/TR+TS strain was associated with a very low PMN, luminol-enhanced, chemiluminescence response, as compared with that of the easily killed type II/no c strain. The present study in combination with the previous work suggest that the type II/TR+TS strain either does not elicit or quenches the PMN metabolic burst response after it is ingested. Cleat and Coid (3) found a type Ic strain that appeared to be phagocytized but that resisted intraphagocytic killing. Whether this resistance is related to the c protein, another component of the cell wall, or a secreted bacterial product is not known.

Poorly killed type II/TR+TS strains are not only ingested as well as easily killed strains but may, under some circumstances, be phagocytized in the absence of serum. The evidence for this was as follows: (i) radiolabeled bacterial uptake in the absence of serum that was 80% of that in the presence of serum (ii) microscopic confirmation that in the absence of serum there were many PMN-associated bacteria at the end of the uptake assay, and (iii) electron microscopic evidence that type II/TR+TS bacteria associated with PMN were in fact internalized within intracellular vacuoles (Fig. 2A). The ligand(s) or receptor(s) involved in the internalization of unopsonized bacteria is not known.

In summary, this study found variations in the opsonic requirements of type II GBS strains, as measured in a bactericidal assay. There appeared to be less C3 and more IgA deposition by immunofluorescence on the poorly killed type II/TR+TS GBS strain as compared with the easily killed type II/no c GBS strain. The type II-specific antibody

concentration was not related to the ability of sera to opsonize type II strains, since 11 of 11 sera promoted a >1 $\log_{10}$ CFUR of a type II/no c GBS strain in the presence or absence of detectable type II-specific antibody. By contrast, only 5 of 11 sera mediated a >1 $\log_{10}$ CFUR of a type II/TR+TS GBS strain, even in the presence of 26.57 μg of type II-specific antibody per ml. Complement was essential for effective, opsonin-mediated CFUR and appeared to be deposited primarily by the classical pathway. For some type II strains, complement alone (in absorbed or hypogammaglobulinemic sera), activated via the classical pathway, appeared to be effective in concert with PMN in promoting a >1 $\log_{10}$ CFUR. In addition, various amounts of type II-specific antibody may have facilitated activation of the alternative pathway, allowing deposition of complement on the bacteria. The difference in PMN killing of the two type II strains appeared to be localized to a defect in intraphagocytic killing rather than a defect in the internalization of the type II/TR+TS strains.

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