INFECTION AND IMMUNITY, Nov. 1992, p. 4801–4808
0019-9567/92/114801-08$02.00/0

Vol. 60, No. 11

Opsonization of *Streptococcus agalactiae* of Bovine Origin by Complement and Antibodies against Group B Polysaccharide

PASCAL RAINARD[1]* AND CHANTAL BOULARD[2]

Laboratoire de Pathologie Infectieuse et d'Immunologie,[1] and Laboratoire de Pathologie Aviaire et de Parasitologie,[2] Institut National de la Recherche Agronomique, Centre de Tours-Nouzilly, 37380 Nouzilly, France

Received 27 May 1992/Accepted 1 September 1992

The contribution of bovine complement and antibodies (Ab) against the group B polysaccharidic antigen (GBA) to the opsonization of *Streptococcus agalactiae* isolated from bovine mastitis cases was investigated by using affinity-purified Ab. GBA-specific Ab were not opsonic by themselves, but in the presence of complement (precolostral calf serum) with an opsonization time of 15 min, they exhibited a dose-dependent opsonic activity in a polymorphonuclear leukocyte chemiluminescence assay. Kinetic studies of the deposition of complement component C3 on protein X-bearing nontypeable (NT/X) strains with an enzyme-linked immunosorbent assay showed that C3 was deposited on bacteria in the absence of Ab but that GBA-specific Ab markedly accelerated the process by reducing the lag phase, which extended up to 15 min when Ab were absent. In the absence of Ab, C3 deposition was inhibited by 5 mM salicylaldoxime or heat treatment at 56°C for 3 min and necessitated Mg^{2+} ions but not Ca^{2+} ions, suggesting that activation of complement was effected by the alternative pathway only. When GBA-specific Ab were added to complement, the inhibitory treatments lost much of their efficacy, suggesting that the classical pathway was recruited. Deposition of C3 on NT/X strains in the absence of Ab induced chemiluminescence and phagocytic killing. With the addition of GBA-specific Ab, the numbers of surviving bacteria were halved ($P < 0.05$) compared with killing in the presence of complement alone. It can be concluded that NT/X strains are activators of the alternative pathway of complement and that GBA-specific Ab reinforce the opsonic efficiency of serum by recruiting the classical pathway and slightly enhancing phagocytic killing.

Streptococcus agalactiae is still a major agent of infection of the bovine mammary gland (30, 45) and produces great losses to dairy workers (9). *S. agalactiae* is also a major cause of serious infections in human neonates and also of puerperal infections (1). Phagocytosis by polymorphonuclear cells (PMN) is considered the major effector of defense in both human and bovine infections by *S. agalactiae* (2, 41). Numerous investigations of phagocytosis of *S. agalactiae* of human origin have led to the conclusion that capsular polysaccharides, which constitute the basis of the serological typing (20), are the main target of opsonic, protective antibodies (23). By contrast, few studies have dealt with the phagocytosis of *S. agalactiae* of bovine origin (17, 25, 26, 37), and still fewer have addressed the specificity of the antibodies which are opsonic (38). Fortunately, the knowledge accumulated in the last 15 years in the medical research field can constitute a valuable working basis for investigations of the opsonization of mastitis-causing *S. agalactiae*. Indeed, bovine and human strains share the same serotypes, which means that, besides the group B polysaccharide antigen (GBA) that is common to all *S. agalactiae* strains, they bear the same capsular polysaccharides. Nevertheless, a sizeable proportion of *S. agalactiae* organisms isolated from mastitis cases are nontypeable (28, 44). Also, the bovine typeable isolates yield smaller amounts of polysaccharidic capsular antigen than do strains of human origin (42). These observations suggest that a thick capsule is not a requisite of *S. agalactiae* pathogenicity for the bovine mammary gland. They also suggest that GBA could be more accessible at the surface of strains of bovine origin than on strains of human origin. This could enable GBA-specific antibodies (Ab) to exert some opsonic activity against mastitis-causing *S. agalactiae*. The present study aimed at testing this possibility by using affinity-purified Ab against GBA and nontypeable as well as encapsulated strains isolated from mastitis cases. The part played by complement in the opsonic process has been showed to be eminent for strains of human origin (3, 4, 12, 13, 19). Since very little information regarding the contribution of complement in the opsonization of mastitis-causing strains is available, particular attention was devoted to this topic in the present study.

MATERIALS AND METHODS

Bacterial strains. Three strains of *S. agalactiae* were initially isolated from cases of bovine mastitis. Strains 24/60 and 443.31 are nontypeable with sera specific for capsular polysaccharides type I to type V but react with anti-protein X serum. They are designated NT/X. Strain 501.21 is a type III/X mastitis isolate. Two type III strains of human origin, 411.05 and 411.19, were used to immunize cows and to prepare GBA, respectively. Each strain was checked for purity on sheep blood agar plates. Strains were serotyped by using specific rabbit antisera and bacterial extracts and the technique of double diffusion in agarose gels (22).

Bovine sera. Precolostral calf serum (PCS) was obtained from a healthy unsuckled calf. The blood was clotted for 1 h at room temperature and centrifuged. The serum was removed, divided in portions, and frozen at −70°C until use. Immune serum (IS) was obtained by immunizing three cows with type III strain 411.05. The serum of these animals taken before immunization was referred to as nonimmune serum. Bacteria were grown overnight in Todd-Hewitt broth,

* Corresponding author.

washed once in saline, and killed by heating for 45 min at 60°C. Cows were injected six times 2 weeks apart with 10^{10} bacteria in incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.) by the subcutaneous route. Serum was obtained 2 weeks after the last injection, and a pool was constituted and stored in portions at −18°C. Before use, IS was heat treated for 30 min at 56°C.

GBA-specific Ab. GBA was purified as described (8) from washed cell walls of a type III strain of human origin (411.19), with some modifications (36). The preparation was virtually devoid of proteins and reacted with rabbit antiserum against GBA but not against type III polysaccharide (36). GBA was immobilized on a matrix to absorb IS and prepare GBA-specific Ab by affinity chromatography. To prepare the affinity column, GBA (26 mg) in 4 ml of distilled water alkalinized with 30 μl of 2 N NaOH was activated by crystals of cyanuric chloride (10 mg) while the pH was monitored with a phenolphthalein indicator. When the solution became colorless (pH 8.0 to 8.2), 3.3 ml of the mixture was pipetted into a tube containing 8 ml of swollen aminohexyl-Sepharose gel (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.1 M phosphate-buffered saline (PBS), pH 8.0, avoiding transfer of cyanuric chloride crystals. The tube was agitated gently end over end at 8°C overnight. The gel was then washed with 0.1 M NaCl in PBS on a glass-sintered filter. The washings were collected, and their carbohydrate content was evaluated by the phenol-sulfuric acid method (10). Slightly more than 5 mg of GBA was coupled to the gel.

Heat-treated (56°C, 30 min) IS (24 ml) was absorbed by being passed over the affinity column. The unretained fraction was collected in 1-ml volumes and considered to be absorbed IS. Then, the column was washed with 20 bed volumes of PBS followed by 2.5 volumes of 1 M NaCl. The retained material was eluted with 0.2 M glycine-HCl buffer, pH 2.8. K_2HPO_4 (1 M) was used to quickly bring the pH back to 7.5. The fractions absorbing at 280 nm were pooled. Assuming an extinction coefficient of 1.37 for bovine immunoglobulins (Ig) (11), a total of 16 mg of Ab was recovered from the 24 ml of IS. These GBA-specific Ab were divided into portions and stored at −18°C either at a concentration of 2 mg/ml in PBS or half diluted in glycerol.

Serum treatments. Inactivation of both complement pathways was accomplished by heating serum at 56°C for 30 min or by chelating both Ca^{2+} and Mg^{2+} ions with 10 mM EDTA (Sigma). The classical complement pathway was inactivated by treating serum with 5 mM $MgCl_2$ and 10 mM ethylene glycol-tetraacetic acid (EGTA) (Sigma) (14). PCS was incubated with bivalent ion chelators for 10 min at room temperature before use. Salicylaldoxime (Sigma) from a stock solution of 0.1 M in 0.15 M NaCl was used at final concentrations of 5 mM and 10 mM; at low concentrations it preferentially inhibits the alternative pathway (31, 32). PCS was depleted of factor B activity by heating at 56°C for 3 min, which inhibits the alternative pathway but leaves an active classical pathway of complement activation (33).

PCS (1.5 ml) was desalted by passage at 4°C through a Sephadex G-25 (Pharmacia) column that had been equilibrated with PBS devoid of Ca^{2+} and Mg^{2+} ions. This desalted PCS was stored at −70°C in small portions until use.

Evaluation of Ab by ELISA. The deposition of Ab on bacteria was monitored by an enzyme-linked immunosorbent assay (ELISA) with whole bacteria (strain 443.31) as antigen, as described previously (38), with some modifications. Gelatin (0.5%) was used instead of skim milk to reduce nonspecific adsorption of protein. The main difference was that monoclonal Ab specific for bovine IgG1, IgG2, and IgM were used to render the ELISA isotype specific (43). The sequence of incubation steps was as follows: (i) appropriate dilution of bovine serum or GBA-specific Ab, (ii) mouse monoclonal Ab specific for bovine Ig isotype, (iii) biotinylated sheep Ab anti-mouse Ig, (iv) streptavidin-horseradish peroxidase (Amersham International plc, Amersham, United Kingdom), (v) substrate of peroxidase. Appropriate dilutions of immune reagents were dispensed in 100-μl samples to triplicate wells. Following each incubation step at 37°C, the plates were washed three times with PBS supplemented with 0.1% Tween 20.

The assay for GBA-specific IgG1, IgG2, and IgM was carried out by the same ELISA procedure except that the antigen was purified GBA coupled to poly-L-lysine (molecular weight, 52,000; Sigma) with cyanuric chloride (Aldrich) as described (16) to make it adherent to the plastic surface of microtiter plates (Immunoplate; Nunc, Roskilde, Denmark).

Assessment of deposition of C3 by ELISA. Microtiter plates were coated with untreated bacteria (strain 443.31) as for the ELISA used to detect Ab against whole bacteria. The sequence of incubation steps, separated by three washings in PBS-Tween 20, was as follows: (i) appropriate dilution in Dulbecco's PBS supplemented with 0.1% gelatin (DPBS-G) of PCS with or without GBA-specific Ab (10 μg/ml), (ii) mouse monoclonal Ab (MD3) specific for the α-chain of bovine C3 (5), (iii) peroxidase-conjugated goat Ab to mouse IgG (Jackson Immunoresearch Laboratory, West Grove, Pa.), (iv) peroxidase substrate. Appropriate dilutions of immune reagents in PBS-Tween 20 were dispensed in 100-μl samples to triplicate wells and incubated for 30 min at 37°C. Wells that received PBS instead of PCS served as blanks.

CL assay (38). Bovine PMN were isolated from the blood of a donor cow (6) and suspended in DPBS-G (2×10^6 cells per ml). Purity and viability (exclusion of trypan blue) both exceeded 95%. The PMN suspension (200 μl) and 100 μl of a 10^{-4} M solution of Luminol (Sigma) were added to 200 μl of opsonized bacteria. The bacterium-to-PMN ratio was about 40. Chemiluminescence (CL) was measured at 38.5°C in a photometer (Luminometer 1251; LKB-Wallac, Turku, Finland) connected to a microcomputer. In each run, test cuvettes containing PMN with either unopsonized bacteria or 5% PCS without bacteria were used. Assays were carried out in duplicate. Most duplicate values differed by less than 5%. CL was expressed in millivolts.

Assay of phagocytic killing. Bacteria were cultivated at 37°C in Todd-Hewitt broth for 18 h. A 1-ml portion of overnight culture was inoculated into 10 ml of fresh Todd-Hewitt broth and incubated at 37°C for 3 h to obtain bacteria in the log phase of growth. Then, bacteria were washed once in PBS and standard suspensions were prepared by diluting the washed organisms in DPBS-G to an optical density (OD) of 0.3 at 600 nm, which corresponds to approximately 2×10^8 CFU/ml. Bacteria were opsonized for 60 min at 37°C by mixing 200 μl of a 10-fold dilution of the standard suspension of bacteria (2×10^7 CFU, 50 μl of PCS, 10 μl of GBA-specific Ab (1 mg/ml), 240 μl of DPBS-G in polypropylene tubes (12 by 75 mm). Then, 200 μl of the PMN suspension (10^7/ml) was added and the reaction mixture was made up to a total volume of 1 ml with DPBS-G. The tubes were incubated for 2 h at 37°C with end-over-end rotation. Immediately before and at the end of incubation, 100-μl samples were made in sterile saline. CFU counts were estimated by streaking 100-μl samples on the surface of predried sheep blood agar plates. Results were expressed as the logarithm of the reduction in CFU numbers and were means from four

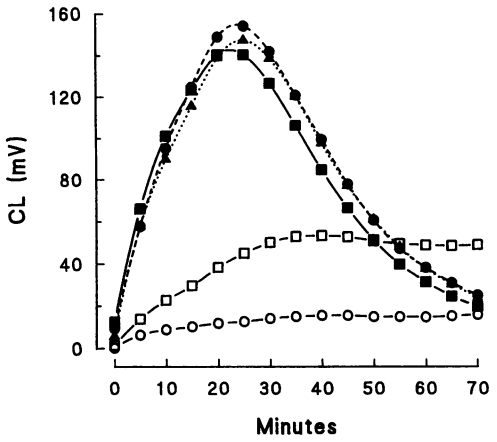

FIG. 1. Time course of the CL response of bovine PMN induced by phagocytosis of *S. agalactiae* 443.31 (NT/X). Bacteria were opsonized for 15 min at 37°C with 5% PCS (○), 10 μg of GBA-specific Ab per ml plus 5% PCS (▲), 2% heat-treated (56°C for 30 min) IS plus 5% PCS (●), 2% heated absorbed IS plus 5% PCS (□), or 2% heated absorbed IS plus 10 μg of GBA-specific Ab per ml plus 5% PCS (■). PMN were then added and the CL responses were recorded.

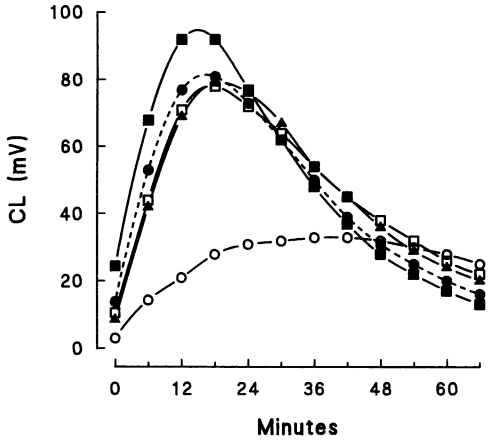

FIG. 2. PMN CL responses to strain 501.21 (type III) opsonized for 15 min at 37°C with 5% PCS (○), 10 μg of GBA-specific Ab per ml plus 5% PCS (▲), 2% heat-treated (56°C for 30 min) IS plus 5% PCS (●), 2% heated absorbed IS plus 5% PCS (□), or 2% heated absorbed IS plus 10 μg of GBA-specific Ab per ml plus 5% PCS (■). PMN were then added and the CL responses were recorded.

separate experiments performed in duplicate. Increases in CFU numbers consistently occurred in control tubes containing PMN and bacteria opsonized with heat-treated PCS.

Microscopic assay of phagocytosis. Phagocytic mixtures were prepared in duplicate tubes as for the bactericidal assay and rotated at 37°C for 1 h. Afterwards, the PMN were washed free of unassociated bacteria by three low-speed centrifugations (100 × *g* for 4 min at 4°C) in cold DPBS. PMN were then resuspended in DPBS, and the suspensions were used to prepare cytocentrifuge smears stained with May-Grünwald-Giemsa reagent. At least 100 PMN were scored microscopically (magnification, ×1,000) for ingestion of bacteria. Results were expressed as the mean number of cell-associated bacteria per PMN.

Statistical analysis. Results were analyzed for statistical significance by unpaired Student's *t* test. *P* values exceeding 0.05 were considered not significant.

RESULTS

Contribution of GBA-specific Ab to the opsonic capacity of bovine serum. The CL assay, which has proved useful in the study of opsonic requirements of group B streptococci (18, 19), was used to assess the opsonization of *S. agalactiae*. The IS obtained by immunizing cows with type III strain 411.05 augmented the CL response of PMN incubated with strain 443.31 (NT/X). With 5% PCS as the source of complement, opsonization with 1% heated serum produced a CL response of 65 ± 4.3 mV before versus 145 ± 5.1 mV after immunization ($P < 0.01$). This reinforcement of CL could not be due to Ab against type III polysaccharide, since strain 443.31 is devoid of this antigen.

To test the possibility that improved CL resulted from the presence of Ab against GBA, the IS was absorbed by being passed over a column of immobilized GBA. The retained Ab were eluted, and their opsonizing activity was evaluated. The CL response was markedly reduced after absorption, and the activity was restored by the addition of affinity-purified Ab to absorbed IS (Fig. 1). These results suggested that a large part of the increased activity of IS was attributable to Ab absorbed on immobilized GBA. The specificity of the effect of absorption on CL was tested by using a strain of *S. agalactiae* (501.21) bearing the type III polysaccharide. With this strain, absorbed IS induced a CL response which was not different from the response obtained with unabsorbed IS (Fig. 2). Interestingly, the purified Ab against GBA were active in the CL assay in the presence of complement (Fig. 2), showing that this capsular antigen-bearing strain of bovine origin was opsonized by Ab directed to GBA. Also, PCS alone demonstrated a nonnegligible activity in the CL assay (Fig. 2).

The contribution of complement and the effect of increasing concentrations of affinity-purified Ab against GBA were assessed by using two nontypeable strains of bovine origin, the prototype NT/X strain 24/60 and strain 443.31. In the absence of opsonins, strain 24/60 elicited a modest CL response which was not augmented by Ab against GBA, whatever the dose tested (Fig. 3). Strain 443.31 did not induce CL in the absence of opsonins, and the addition of Ab against GBA remained without effect (Fig. 3). In the presence of 5% PCS, there was a dose-response curve of CL with the two strains, which peaked at a concentration of 5 μg of Ab per ml and ended by a plateau (Fig. 3). At the concentration of 5% and with an incubation time of 15 min, PCS induced only a slight increase in CL over the CL provoked by unopsonized bacteria but enabled GBA-specific Ab to express a CL-promoting activity. As the role of complement appeared to be crucial in the opsonization of NT/X strains of *S. agalactiae*, investigations of the deposition on bacteria of the potentially opsonic C3 component were undertaken.

Deposition of opsonins on NT/X strains of bovine origin. The deposition of C3 was studied by using the whole-cell ELISA (strain 443.31) and a monoclonal Ab specific to bovine C3. Preliminary experiments showed that in the presence of 1% IS and with an incubation time of 30 min, increasing concentrations of PCS resulted in increased ELISA responses up to concentrations of 10 to 15%. PCS was used at a concentration of 10% to investigate the kinetics of deposition of C3 on strain 443.31 and the influence of Ab against GBA on this phenomenon (Fig. 4). It

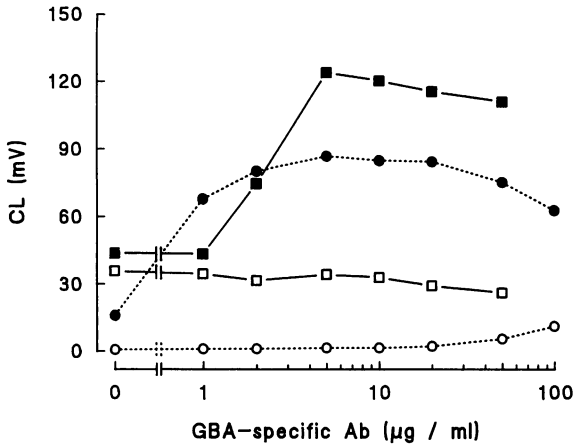

FIG. 3. Peaks of PMN CL responses to the NT/X strains 24/60 (□, ■) and 443.31 (○, ●) either unopsonized or opsonized for 15 min at 37°C with increasing concentrations of either GBA-specific Ab (open symbols) or GBA-specific Ab plus 5% PCS (closed symbols).

appeared that PCS alone produced a deposition of C3 which was of the same magnitude as that obtained in the presence of Ab but with different kinetics. The striking difference was the lag phase preceding the onset of deposition of C3. At 15 min of incubation, the time used in the CL experiments reported above, about 40% of maximal C3 ELISA response occurred in the presence of Ab against GBA, whereas virtually no C3 was detectable when PCS operated alone. Past this delay, deposition of C3 in the absence of Ab took place and reached a maximum at 60 min of incubation. Comparable results were obtained with strain 24/60 (data not shown).

The deposition of Ab from the different sera used in this study was evaluated by using the whole-cell ELISA with strain 443.31 as the antigen (Table 1). PCS was virtually devoid of Ab. Absorbed IS lost most of its activity in the IgG1 and IgM isotypes but retained most of its activity in the IgG2 isotype. Ab specific to GBA were also sought in the

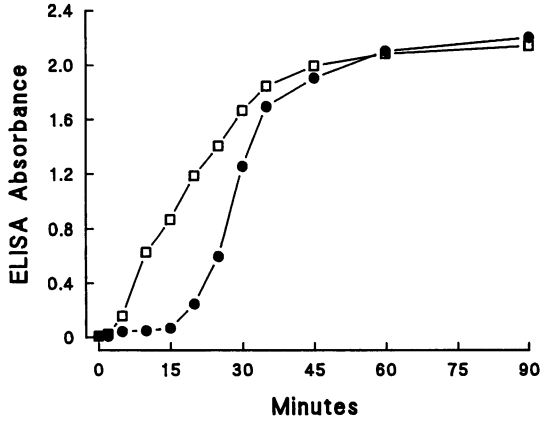

FIG. 4. Time course of C3 deposition on *S. agalactiae* 443.31. Bacteria were incubated at 37°C in a warm room (37°C) with 10% PCS supplemented (□) or not (●) with 10 μg of GBA-specific Ab per ml. C3 deposition was measured by ELISA in microtiter plates. To each incubation time was allocated a vertical row of the microtiter plate. The rows were washed at given times and the plate was allowed to incubate until the last row was used.

TABLE 1. Results of isotype-specific ELISA to assess the deposition of Ab on strain 443.31 and to detect GBA-specific Ab in the opsonin sources used in opsonization experiments[a]

ELISA antigen	Ig isotype	OD with:				
		10% PCS	1% NIS	1% IS	1% abs-IS	10 μg of GBA-specific Ab per ml
Strain 443.31 (NT/X)	G1	0.03	0.54	1.83	0.30	1.27
	G2	0.08	1.55	2.65	2.51	1.56
	M	0.02	0.34	0.54	0.15	0.21
GBA	G1	0.03	0.61	1.80	0.02	1.54
	G2	0.04	0.68	2.20	0.28	1.56
	M	0.02	0.29	0.28	0.04	0.18

[a] Comparisons of ODs are valid only for results obtained with the same antigen and the same isotype-specific second Ab (within horizontal rows). Values are means from two independent experiments performed in triplicate. NIS, nonimmune serum; abs-IS, IS absorbed with GBA.

sources of Ab used in this study, by using purified GBA coupled to poly-L-lysine as the antigen in the ELISA (Table 1). Before immunization, bovine serum contained Ab against GBA, but immunization markedly reinforced the Ab activity. Absorbed serum lost most of its activity, this time also in the IgG2 isotype, suggesting that the remaining IgG2 activity detected with the whole-bacterium ELISA was not directed to GBA but to other surface antigens. The fraction of IS retained by and eluted from the affinity column, and referred to as GBA-specific Ab, demonstrated an Ab activity against both purified GBA and whole bacteria in the IgG1 and IgG2 isotypes, with a low activity in the IgM isotype.

Contribution of the classical and the alternative pathways to deposition of C3. The contribution of the two main pathways of activation of complement was investigated with the ELISA measuring the deposition of C3 on whole bacteria (Table 2). Various inhibiting treatments were applied to PCS, the source of complement. Treatment of PCS with 10 mM EDTA or heat treatment at 56°C for 30 min abolished the ELISA response. These treatments are known to inactivate the two pathways of complement activation. This result also demonstrated that C3, which is not destroyed by these treatments, did not absorb passively to bacteria and that the ELISA gives negative results in the absence of an active cascade of complement activation. Heat treatment of

TABLE 2. ODs in an ELISA measuring the deposition of C3 on *S. agalactiae* strains 24/60 and 443.31 (NT/X), using whole bacteria as antigen[a]

Inhibitor	OD (SEM) for 24/60 with:		OD (SEM) for 443.31 with:	
	PCS	PCS + Ab	PCS	PCS + Ab
None	1.72 (0.09)	1.90 (0.05)	1.68 (0.04)	1.80 (0.06)
EDTA, (10 mM)	0.01 (0.01)	0.12 (0.02)	0.01 (0.01)	0.12 (0.02)
56°C, 30 min	0.01 (0.01)	0.28 (0.05)	0.01 (0.01)	0.15 (0.04)
Mg-EGTA (10 mM)	0.07 (0.02)	0.14 (0.04)	0.05 (0.04)	0.07 (0.02)
56°C, 3 min	0.31 (0.05)	1.60 (0.08)	0.09 (0.02)	1.28 (0.06)
Salicylaldoxime				
5 mM	0.04 (0.01)	1.26 (0.12)	0.10 (0.01)	1.10 (0.11)
10 mM	0.01 (0.01)	1.20 (0.07)	0.02 (0.01)	0.85 (0.06)

[a] PCS (10%) was used as the source of complement, with or without affinity-purified Ab against GBA (10 μg/ml), and various inhibiting treatments of complement were applied. Opsonization took place at 37°C for 45 min. Results from three distinct experiments performed in triplicate are given.

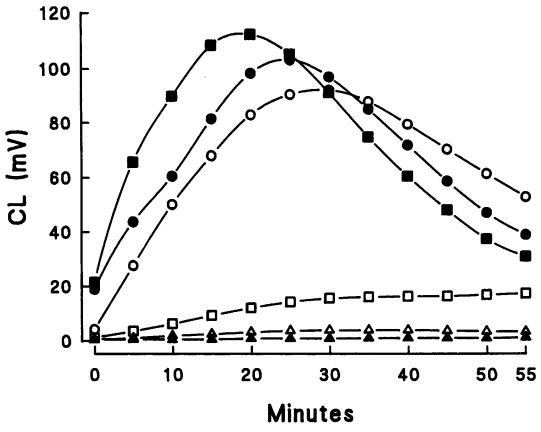

FIG. 5. PMN CL responses to strain 443.31 unopsonized (▲) or opsonized at 37°C with 5% PCS for either 15 min (□) or 45 min (■), 5% PCS plus 10 μg of GBA-specific Ab per ml for either 15 min (○) or 45 min (●), or 10 μg of GBA-specific Ab per ml alone (△).

PCS at 56°C for 3 min, which destroys mainly factor B of the alternative pathway (33), resulted in almost complete abrogation of the ELISA response in the absence of Ab, whereas in its presence the decrease was moderate. Treatment of PCS with salicylaldoxime, which has been reported to inhibit essentially the alternative pathway when used at low concentrations (31, 32), abolished the ELISA response of the Ab-independent opsonization. In the presence of Ab there was some inhibition, which was greater with the higher concentration of salicylaldoxime. These results suggest that the alternative pathway was responsible for C3 deposition effected by PCS. Nevertheless, chelation of divalent ions of PCS with Mg-EGTA, which is used as an inhibitor of the Ca-dependent activity of C1 (14), provoked a major reduction in ELISA activity (Table 2), thus contradicting the exclusive participation of the alternative pathway in the absence of Ab. Since it has been reported that Mg-EGTA could block the alternative pathway of bovine complement (21), the participation of Ca^{2+} and Mg^{2+} in C3 deposition was investigated with desalted PCS. PCS deprived of Ca^{2+} and Mg^{2+} by passage over a desalting column (Sephadex G-25) was used to examine the effect on the deposition of C3 on bacteria (strain 443.31) of adding $CaCl_2$ (final concentration, 0.15 mM) and $MgCl_2$ (final concentration, 0.5 mM), together or singly. Whole and desalted PCSs (10%) were incubated for 1 h at 37°C. The ELISA ODs were 1.75 and 0.01, respectively. The addition of $CaCl_2$ did not restore C3 deposition, whereas the addition of $MgCl_2$ did (OD, 1.69). The addition of both cations yielded an ELISA OD of 1.84. This result demonstrated that C3 deposition necessitated only $MgCl_2$ ions, incriminating the alternative pathway of complement activation.

Relationship between deposition of C3 and phagocytosis. A limiting concentration of complement, i.e., 5% PCS, was used to show the influence on the CL response of PMN mixed with bacteria (strain 443.31) of both the opsonization incubation time and the addition of Ab against GBA (Fig. 5). Unopsonized bacteria or bacteria opsonized with Ab alone did not stimulate PMN. Opsonization with PCS for 15 min elicited only a slight CL response, which contrasted with the good CL response obtained when PCS was supplemented with Ab against GBA or when PCS was incubated for 45 min. With incubation for 45 min, the addition of Ab did not augment the CL peak response. Thus, an optimal CL response could be obtained with complement alone, but, under limiting conditions, the presence of GBA-specific Ab reinforced markedly the CL response. Taken together, these results showed a good relationship between the deposition of C3 and the CL response of PMN.

The CL assay was used as an indirect assay of phagocytosis. The bactericidal activity of PMN mixed with bacteria opsonized under limiting concentration and incubation time (15 min) of complement was determined in order to assess whether the CL responses correlated with the opsonophagocytic killing activity of PMN. Figure 6 shows that with strain 443.31, a reduction in bacterial numbers was obtained only when 5% PCS was supplemented with GBA-specific Ab. With strain 24/60, some killing was obtained at 120 min of incubation with PMN when bacteria were opsonized with PCS alone, which can be related to the modest CL response obtained under the same conditions of opsonization (Fig. 3). Nevertheless, the supplementation of PCS with GBA-specific Ab improved phagocytic killing. These results showed that the cooperative effect of PCS and GBA-specific Ab

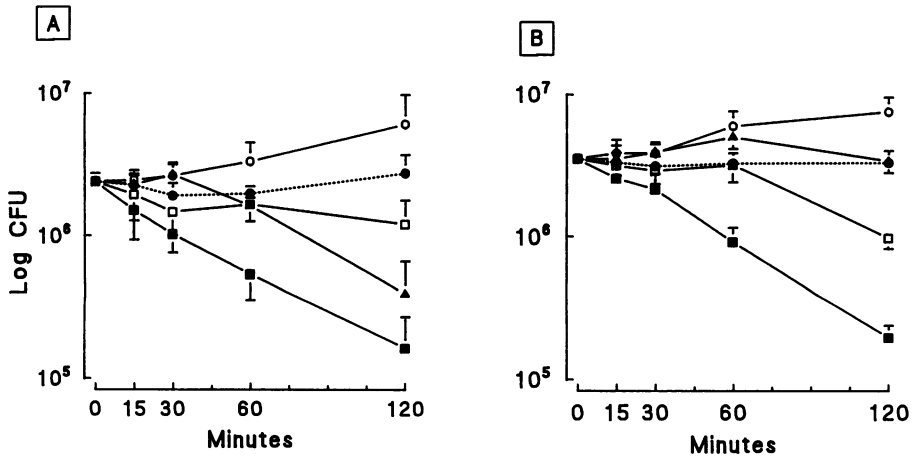

FIG. 6. Time course of phagocytic killing by bovine PMN of *S. agalactiae* 24/60 (A) or 443.31 (B) opsonized for 15 min at 37°C with 5% PCS (▲), 10 μg of GBA-specific Ab per ml (□), or 5% PCS plus 10 μg of GBA-specific Ab per ml (■). Controls included unopsonized bacteria (●) and bacteria opsonized with PCS plus Ab but without addition of PMN (○).

TABLE 3. Determination of numbers of PMN-associated bacteria after treatment with GBA-specific Ab with or without complement[a]

Treatment of bacteria	No. (SEM) of strain	
	443.31	24/60
GBA-specific Ab		
10 μg/ml	0.31 (0.06)	0.24 (0.04)
25 μg/ml	0.35 (0.06)	0.38 (0.07)
10 μg of Ab per ml + 10% PCS	12.7 (0.82)	13.5 (1.88)

[a] Values are from two distinct experiments performed in duplicate.

demonstrated in the CL assay was also operating in the phagocytic killing assay.

If CL correlates with phagocytic killing, then killing of bacteria opsonized with PCS for 45 min or longer should occur. It would also be interesting to see whether, under conditions favorable to deposition of complement, GBA-specific Ab could improve phagocytic killing. The phagocytic killing assay revealed that opsonization with PCS alone enabled PMN to kill bacteria: the reductions in $\log_{10}$ CFU were (means ± standard errors of the means) 0.85 ± 0.04 and 0.90 ± 0.07 for strains 24/60 and 443.31, respectively. This demonstrated that deposition of C3 efficiently stimulated PMN without the need for Ab. The addition of GBA-specific Ab to the complement source augmented the efficacy of phagocytic killing ($P < 0.05$): $\log_{10}$ reductions in CFU were, respectively, 1.11 ± 0.11 and 1.26 ± 0.12 for strains 24/60 and 443.31, i.e., approximately a 50% reduction in CFU. Nevertheless, even in the presence of Ab, the proportion of surviving bacteria was substantial since the reduction in bacterial numbers remained far from 2 $\log_{10}$ units (i.e., 1% survival).

The possibility that Ab-coated bacteria adhered to PMN but were not ingested was explored by a microscopic assessment of phagocytosis. Bacteria (strains 24/60 and 443.31) opsonized with 10 or 25 μg of GBA-specific Ab per ml were seldom associated with PMN, whereas with an addition of complement to Ab, an average of 13 bacteria per PMN were recorded (Table 3). This showed that GBA-specific Ab were unable to induce adherence of *S. agalactiae* to PMN.

DISCUSSION

Initially devised to define the role of GBA-specific Ab in the opsonization of mastitis-causing strains of bovine origin, the present study also yielded useful information on the role played by complement. No attempt was made in this study to characterize the fragments of C3 (C3b, C3bi, and C3d in various combinations) deposited on bacteria, and they are simply referred to as C3. The NT/X strains used in this study behaved like particulate activators of the alternative pathway. Direct complement activation, leading to C3 deposition on bacteria, took place in the absence of detectable Ab through the alternative pathway of complement activation exclusively, as shown by the dramatic effect of inhibitors of this pathway and by the requirement for Mg^{2+} but not Ca^{2+} ions. The spontaneous activation of the alternative pathway by NT/X strains is different from activation by encapsulated strains of human origin, types Ia and III, which requires a sufficient amount of Ab (3, 13). Deposition of C3 through the alternative pathway enabled PMN to ingest and kill the coated bacteria, demonstrating that C3 was an efficient opsonin. The CL response of phagocytosing PMN was well related to the efficacy of C3 deposition with the different concentrations and incubation times used in this study, demonstrating a good stimulation of phagocytes in the absence of Ab. The absence of classical pathway activation suggests that the NT/X strains, unlike type Ia group B streptococci of human origin (3, 12), do not interact directly with C1q.

In the study of Raff et al. (35) demonstrating the opsonic activity of human GBA-specific monoclonal Ab, a source of complement was systematically incorporated in the opsonizing system. Given the general resistance of the surface of human strains to activation of complement, specific Ab are crucial to opsonization (4, 13, 18, 19). It must be noted that GBA-specific Ab are not generally considered to play a major role in protection against infection. Rabbit Ab to GBA were not protective in a mouse model (24) and were not active in a CL assay of phagocytosis (19). In another study, they behaved as poor opsonins compared with Ab against capsular polysaccharide, though they allowed some bactericidal killing by PMN (4). The opsonic activity demonstrated by human monoclonal Ab against GBA (35) prompted in part the present work, considering that bovine typeable *S. agalactiae* bear smaller amounts of capsular antigen than do isolates of human origin (42).

This study specified the contribution of GBA-specific Ab to opsonization of bovine isolates. Ab proved unable to opsonize alone. Their inability to even promote adherence of *S. agalactiae* to PMN was somewhat disconcerting since adherence to phagocytes possessing receptors for IgG2 (27) could be expected from bacteria coated with Ab rich in IgG2. The main effect of Ab was to facilitate activation and binding of C3 to the bacteria by recruiting the classical pathway of complement activation. Deposition of C3 occurred in the presence of salicylaldoxime, a preferential inhibitor of the alternative pathway (31, 32), or after heat treatment at 56°C for 3 min, which destroys bovine factor B (33). A striking effect of the addition of GBA-specific Ab to the opsonic system was the modification of the kinetics of C3 deposition. With complement alone, the protracted lag phase preceding the onset of C3 deposition was an important feature of the process. The duration of the initial lag phase is a characteristic of the alternative pathway (34), so the virtual suppression of the lag phase when GBA-specific Ab were added can be considered to result from the recruitment of the classical pathway, although the possibility of a reinforcement of the alternative pathway by Ab was not investigated in this study. Another effect of GBA-specific Ab was the improvement of phagocytic killing. Whether this resulted from the optimization of the deposition of C3 on bacteria, from the recruitment of Fc receptors of the phagocytes, or from another mechanism remains to be determined.

It is worth mentioning that the divalent cation chelator EGTA was not useful in discriminating between the two pathways of complement activation. A widely used inactivator of the classical pathway, EGTA, behaved in the present study like EDTA, which inhibits classical and alternative pathways. Mg-EGTA has been reported to inhibit complement alternative pathway-mediated killing of mycoplasmas and of *Escherichia coli* by calf serum but not by human serum (21), suggesting a different effect of EGTA according to the source of serum. This might be related to the greater enhancing effect of Mg^{2+} than of Ca^{2+} on the interaction of bovine complement with substrate compared with the effect of the two cations on guinea pig and human complement (15). But this is probably not the only contributing factor, since Mg-EGTA can be used to selectively

inhibit classical pathway-mediated lysis of erythrocytes by bovine complement (32), suggesting that some properties of the complement-activating surface are also playing a part.

The importance of Ab cannot be dismissed on the basis of the Ab-independent deposition of C3 on a bacterial surface. First, killing of C3-coated bacteria could be increased by additional opsonization by Ab directed to outer surface antigens, as it was when GBA-specific Ab were added to complement. Second, the biological fluid in which opsonization and phagocytosis take place in mastitis cases is milk. The point is that bovine milk has a very low complement activity (40). In fact, complement activity in milk has been revealed through C3bi conglutinogenic activity (7) and by hemolytic and bactericidal assays (29, 39), not through the deposition of C3 on bacteria. Thus, whether normal milk can enable enough C3 deposition to render bacteria suitable for phagocytosis by PMN remains to be shown before the role of Ab can be fully evaluated. Also, Ab may be able to act in the absence of complement. Recently, we have shown that heat-treated serum of cows immunized with protein X of *S. agalactiae* was still more opsonizing than untreated preimmune serum (38). This could indicate that GBA is not the best target of opsonins on mastitis-causing *S. agalactiae*, as far as phagocytosis is to take place in a medium in which complement activity is reduced, as it is in milk from an uninflamed mammary gland. The opsonic activity of antibodies directed to capsular polysaccharides or surface proteins, such as protein X, deserves investigation. GBA-specific Ab present the great advantage of reacting with every strain and are able to reinforce the phagocytic killing, but they are dependent on complement activity. This indicates that GBA-specific Ab are likely to be useful as soon as inflammation develops, with the supply of complement afforded by exudation of plasma (29, 39).

ACKNOWLEDGMENTS

We thank B. Poutrel and P. Sarradin for their encouragement and advice since the beginning of this work and D. Van Zaane for the gift of monoclonal Ab against bovine Ig.

REFERENCES

1. **Baker, C. J.** 1980. Group B streptococcal infections. Adv. Intern. Med. **25**:475–501.
2. **Baker, C. J., and D. L. Kasper.** 1985. Group B streptococcal vaccines. Rev. Infect. Dis. **7**:458–467.
3. **Baker, C. J., D. L. Kasper, and C. E. Davis.** 1982. Antibody-independent classical pathway-mediated opsonophagocytosis of type Ia, group B *Streptococcus*. J. Clin. Invest. **69**:394–404.
4. **Baltimore, R. S., D. L. Kasper, C. J. Baker, and D. K. Goroff.** 1977. Antigenic specificity of opsonophagocytic antibodies in rabbit anti-sera to group B streptococci. J. Immunol. **118**:673–678.
5. **Boulard, C.** 1989. Degradation of bovine C3 by serine proteases from parasites *Hypoderma lineatum* (Diptera, Oestridae). Vet. Immunol. Immunopathol. **20**:387–398.
6. **Carlson, G. P., and J. J. Kaneko.** 1973. Isolation of leukocytes from bovine peripheral blood. Proc. Soc. Exp. Biol. Med. **142**:853–856.
7. **De Cueninck, B. J.** 1979. C_{142} complement activity and conglutinogen in bovine milk. Int. Arch. Allergy Appl. Immunol. **59**:323–327.
8. **De Cueninck, B. J., G. D. Shockman, and R. M. Swenson.** 1982. Group B, type III streptococcal cell wall: composition and structural aspects revealed through endo-*N*-acetylmuramidase-catalyzed hydrolysis. Infect. Immun. **35**:572–582.
9. **Dobbins, C. N., Jr.** 1977. Mastitis losses. J. Am. Vet. Med. Assoc. **170**:1129–1132.
10. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–358.
11. **Duncan, J. R., B. N. Wilkie, F. Hiestand, and A. J. Winter.** 1972. The serum and secretory immunoglobulins of cattle: characterization and quantitation. J. Immunol. **108**:965–976.
12. **Eads, M. E., N. J. Levy, D. L. Kasper, C. J. Baker, and A. Nicholson-Weller.** 1982. Antibody-independent activation of C1 by type Ia group B streptococci. J. Infect. Dis. **146**:665–672.
13. **Edwards, M. S., A. Nicholson-Weller, C. J. Baker, and D. Kasper.** 1980. The role of specific antibody in alternative complement pathway-mediated opsonophagocytosis of type III, group B streptococcus. J. Exp. Med. **151**:1275–1287.
14. **Fine, D. P., S. R. Marney, D. G. Colley, J. S. Sergent, and R. M. Desprez.** 1972. C3 shunt activation in human serum chelated with EGTA. J. Immunol. **109**:807–809.
15. **Fong, J. S. C., L. H. Muschel, and R. A. Good.** 1971. Kinetics of bovine complement. I. Formation of a lytic intermediate. J. Immunol. **107**:28–33.
16. **Gray, B. M.** 1979. ELISA methodology for polysaccharide antigens: protein coupling of polysaccharides for adsorption to plastic tubes. J. Immunol. Methods **28**:187–192.
17. **Hahn, G., and A. Tolle.** 1981. Vergleichende Untersuchungen zur Charakterisierung von aus Mensch und Rind isolierten Gruppe B-Streptokokken (*Sc. agalactiae*) mit Hilfe eines Bakterizidie-Tests. Zentralbl. Bakteriol. Abt. 1 Orig. A **249**:15–23.
18. **Hastings, M. J. G., and C. S. F. Easmon.** 1981. Variations in the opsonic requirements of group B Streptococcus type III. Br. J. Exp. Pathol. **62**:519–525.
19. **Hemming, V. G., R. T. Hall, P. G. Rhodes, A. O. Shigeoka, and H. R. Hill.** 1976. Assessment of group B streptococcal opsonins in human and rabbit serum by neutrophil chemiluminescence. J. Clin. Invest. **58**:1379–1387.
20. **Henrichsen, J., P. Ferrieri, J. Jelinkova, W. Köhler, and W. R. Maxted.** 1984. Nomenclature of antigens of group B streptococci. Int. J. Syst. Bacteriol. **34**:500.
21. **Howard, C. J.** 1976. Variation in the susceptibility of bovine mycoplasmas to killing by the alternative complement pathway in bovine serum. Immunology **41**:561–568.
22. **Jensen, N. E.** 1979. Production and evaluation of antisera for serological type determination of group B streptococci by double diffusion in agarose gel. Acta Pathol. Microbiol. Scand. Sect. B **87**:77–83.
23. **Kasper, D. L.** 1986. Bacterial capsule. Old dogmas and new tricks. J. Infect. Dis. **153**:407–415.
24. **Lancefield, R. C., M. McCarty, and W. N. Everly.** 1975. Multiple mouse-protective antibodies directed against group B streptococci. J. Exp. Med. **142**:165–179.
25. **Mackie, D. P., D. A. Pollock, and E. F. Logan.** 1982. In vitro bactericidal assay of bovine polymorphonuclear leucocytes against a group B *Streptococcus*. Res. Vet. Sci. **33**:240–242.
26. **Mackie, D. P., D. A. Pollock, and E. F. Logan.** 1985. The opsonic activity of whey and sera from heifers experimentally infected with *Streptococcus agalactiae*. Br. Vet. J. **141**:349–354.
27. **McGuire, T. C., A. J. Musoke, and T. Kurtti.** 1979. Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages, neutrophils and skin. Immunology **38**:249–256.
28. **Morrison, J. R. A., and C. L. Wright.** 1984. *Streptococcus agalactiae* serotypes in the southwest of Scotland. Vet. Rec. **115**:439.
29. **Mueller, R. E., E. J. Carroll, and L. Panico.** 1982. Complement C3 levels and hemolytic activity in normal and mastitic whey. Zentralbl. Veterinaermed. Reihe B **29**:99–106.
30. **Oliver, S. P., and B. A. Mitchell.** 1984. Prevalence of mastitis pathogens in herds participating in a mastitis control program. J. Dairy Sci. **67**:2436–2440.
31. **Osler, A. G., and A. L. Sandberg.** 1973. Alternative complement pathways. Prog. Allergy **17**:51–92.
32. **Pang, A. S. D., and W. P. Aston.** 1977. Alternative complement pathway in bovine serum: lysis of human erythrocytes. Am. J. Vet. Res. **38**:355–359.
33. **Pang, A. S. D., and W. P. Aston.** 1978. The alternative comple-

ment pathway in bovine serum: the isolation of a serum protein with factor B activity. Immunochemistry **15:**529–534.
34. **Pangburn, M. K., R. D. Schreiber, and H. J. Müller-Eberhard.** 1983. C3b deposition during activation of the alternative complement pathway and the effect of deposition on the activating surface. J. Immunol. **131:**1930–1935.
35. **Raff, H. V., P. J. Siscoe, E. A. Wolff, G. Maloney, and W. Shuford.** 1988. Human monoclonal antibodies to group B *Streptococcus*. Reactivity and in vivo protection against multiple serotypes. J. Exp. Med. **168:**905–917.
36. **Rainard, P.** 1992. Isotype antibody response in cows to *Streptococcus agalactiae* group B polysaccharide-ovalbumin conjugate. J. Clin. Microbiol. **30:**1856–1862.
37. **Rainard, P., Y. Lautrou, and B. Poutrel.** 1988. Ingestion and killing of *Streptococcus agalactiae* by bovine granulocytes in the presence of natural opsonins. Vet. Microbiol. **18:**41–50.
38. **Rainard, P., Y. Lautrou, P. Sarradin, and B. Poutrel.** 1991. Protein X of *Streptococcus agalactiae* induces opsonic antibodies in cows. J. Clin. Microbiol. **29:**1842–1846.
39. **Rainard, P., B. Poutrel, and J. P. Caffin.** 1984. Assessment of hemolytic and bactericidal complement activities in normal and mastitic bovine milk. J. Dairy Sci. **67:**614–619.
40. **Reiter, B., and J. D. Oram.** 1967. Bacterial inhibitors in milk and other biological fluids. Nature (London) **216:**328–330.
41. **Schalm, O. W., J. Lasmanis, and E. J. Carroll.** 1967. Experimental *Streptococcus agalactiae* mastitis in cattle: attempts to superimpose the organism in lactating glands with experimentally induced sterile inflammation. Am. J. Vet. Res. **28:**685–695.
42. **Straus, D. C., M. K. Yeung, D. L. Durham, and S. J. Mattingly.** 1982. Lack of virulence of bovine type III *Streptococcus agalactiae* strains for mice correlates with reduced in vitro production of extracellular type-specific antigen. Curr. Microbiol. **7:**251–256.
43. **Van Zaane, D., and J. Ijzerman.** 1984. Monoclonal antibodies against bovine immunoglobulins and their use in isotype-specific ELISAs for rotavirus antibody. J. Immunol. Methods **72:**427–441.
44. **Wibawan, I. W. T., and C. Lämmler.** 1990. Properties of group B streptococci with protein surface antigens X and R. J. Clin. Microbiol. **28:**2834–2836.
45. **Wilson, C. D., and M. S. Richards.** 1980. A survey of mastitis in the British dairy herd. Vet. Rec. **106:**431–435.