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Protein X of *Streptococcus agalactiae* Induces Opsonic Antibodies in Cows

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Protein X of *Streptococcus agalactiae* is a surface protein frequently associated with strains isolated from cases of mastitis of dairy cows. By immunizing cows with purified protein X, we obtained an antibody response which was restricted to X-bearing strains of *S. agalactiae* in a whole-cell enzyme-linked immunosorbent assay. This response resulted in an increase in the opsonic activity of serum for strains bearing protein X, as assessed through the augmentation of the chemiluminescence response of phagocytosing polymorphonuclear cells and through an increased ingestion of bacteria, although the proportion of ingested bacteria which were killed (about 60%) remained unchanged. Protein X behaved as a target of opsonins and, as such, could be a protective antigen worth incorporating in a vaccine against *S. agalactiae* mastitis.

Streptococcus agalactiae is one of the main agents responsible for mastitis in cattle (16) and an important cause of human perinatal infections (20). Protein X of *S. agalactiae* is a surface protein (18) useful in the typing of strains of bovine origin (12). Epidemiological studies have identified protein X in a large proportion of strains isolated from cases of bovine mastitis (7, 10, 11). Despite its high prevalence, few studies have dealt with this molecule, and information is not available concerning its role in the pathogenicity of mastitis and the immune protection it could induce.

In the present study, we used purified protein X (9) to investigate its immunogenicity in cows. The effect of immunization on the opsonic activity of serum was also measured in order to assess the immunological significance of protein X.

MATERIALS AND METHODS

Bacterial strains. Seventeen strains of *S. agalactiae* were used in this study either to assess the specificity of the antibody response of immunized cows or to evaluate the opsonic activity of sera (Table 1). Strain 443.31 was used to produce protein X (9). Before use, strains were subcultured in Todd-Hewitt broth (THB). Each strain was checked for purity on sheep blood agar plates, and Gram staining, catalase test, and grouping with sensitized latex (Slidex Strepto-Kit; Biomérieux, Marcy l'Etoile, France) were performed. Strains were serotyped by using specific rabbit antisera and bacterial extracts (8) and the technique of double diffusion in agarose gels (6).

For use in phagocytosis assays, bacteria were grown overnight in THB. Two milliliters of this culture was inoculated in 8 ml of fresh THB and incubated at 37°C for 2 h. Bacteria were harvested by centrifugation at 1,500 × *g* for 15 min, washed once in Dulbecco's phosphate-buffered saline (PBS), and resuspended to the desired concentration in PBS supplemented with 0.1% gelatin (PBS-G).

Sera. Three lactating cows (numbered 560, 601, and 603) belonging to the experimental herd at our laboratory were used. None of them had previously had mastitis caused by *S. agalactiae*. They received two subcutaneous injections, 74 days apart, of 300 μg of purified protein X (9) emulsified in incomplete Freund adjuvant. A final dose of antigen (25 μg) was administered into two quarters of each cow through the teat canal 30 days after cessation of milking. Blood samples were collected immediately before the first injection and at intervals thereafter (Fig. 1). The blood was allowed to clot and centrifuged. The resulting sera were diluted 1/2 in glycerol and stored at −20°C. A pool of immune serum (IS) was constituted by mixing the sera collected from the three immunized cows on day 87 postinjection. This pool of IS served as a reference serum for the enzyme-linked immunosorbent assay (ELISA) titration. Precolostral calf serum (PCS), obtained from a newborn unsuckled calf and stored in portions at −70°C, served as the source of complement.

ELISA. The antibody response of immunized cows was monitored by an ELISA with whole bacteria as antigen. Flat-bottom 96-well microtiter plates (Immunoplate; Nunc, Roskilde, Denmark) were coated with heat-killed bacteria of strain 443.31. Bacteria were suspended in Dulbecco's PBS (optical density at 600 nm [OD_{600}] of 0.1), dispensed in 0.1-ml samples into the wells, and incubated overnight at 4°C. Under these conditions, bacteria adhere firmly to the polystyrene bottom (14). The plates were washed and incubated for 30 min at 37°C with 5% powdered skim milk in PBS (wt/vol) to reduce nonspecific adsorption of proteins. Following each incubation step, the plates were washed three times with PBS supplemented with 0.1% (vol/vol) Tween 20 (Sigma) (PBS-T). Reagents were diluted in PBS-T supplemented with 0.5% (wt/vol) gelatin (PBS-TG). The sequence of incubation steps was as follows: (i) appropriate dilution of the bovine serum being tested, (ii) rabbit antiserum to bovine immunoglobulin, (iii) peroxidase-conjugated goat antibody to rabbit immunoglobulin G (heavy and light chains) (Jackson Immunoresearch Laboratories, West Grove, Pa.), and (iv) 52 mM 2,2'-azino-di-(3-ethylbenzthiazoline-sulfonate) (Boehringer Gmbh, Mannheim, Germany) in 0.1 M citrate buffer (pH 4.2) with 0.075% hydrogen peroxide added just before use. The enzymatic reaction was stopped, after about 30 min at room temperature, with 50 μl of 1 mM NaN_3. The OD_{414} was read with a Titertek Multiscan spectrophotometer (Flow Laboratories, McLean, Va.). Wells that received PBS instead of bovine serum served as blanks.

The antibody content of sera was quantified with reference

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TABLE 1. *S. agalactiae* strains used in this study

Origin and strain	Serotype	Utilization[a]	Source[b]
Human			
411.01	Ia	ELISA	IP
411.04	II	OPSO	IP
411.05	III	OPSO	IP
419.12	II	ELISA	IB
419.19	II	ELISA	IB
445.05	III/R	ELISA, OPSO	IH
445.06	NT/R	ELISA	IH
Bovine			
46.70	III	ELISA	PII
48.17	NT/X	ELISA	PII
78.32	II	ELISA	PII
89.01	NT/X	ELISA	PII
401.29	NT/X	ELISA	PII
411.06	NT/R	ELISA, OPSO	IP
411.07	NT/X	OPSO	IP
443.19	II	ELISA	PII
443.21	II/X	OPSO	PII
443.31	NT/X	ELISA, OPSO	IH

[a] ELISA used to assess the specificity of antibody response. OPSO, opsonization studies.
[b] IB, Institut Bactériologique, Strasbourg, France; IH, Institut für Hygiene, Kiel, Germany; IP, Institut Pasteur, Paris, France; PII, Laboratoire de Pathologie Infectieuse et d'Immunologie, Institut National de la Recherche Agronomique, Nouzilly, France.

to the pool of IS which was arbitrarily assigned a titer of 10,000 U (2). Six dilutions of this serum were incorporated in each microtiter plate to establish its titration curve. The OD values given by the tested sera were referred to this curve to convert the ELISA readings into arbitrary units. In comparison, PCS had a titer of 10 arbitrary units.

To assess the specificity of the antibody response of the immunized cows, the microtiter plates were coated with a set of group B streptococci (GBS) strains of different serotypes (Table 1).

CL assay. Bovine polymorphonuclear leukocytes (PMN) were isolated from the blood of a donor cow (4) and suspended in PBS-G. The chemiluminescence (CL) of phagocytosing PMN was measured as previously described (17). Briefly, bacteria were opsonized for 15 min at 37°C with 2% heat-treated (at 56°C for 45 min) bovine serum with or without 5% PCS as a source of complement devoid of antibodies to GBS (15). They were then mixed with PMN and Luminol (Sigma). The bacteria-to-PMN ratio was about 40. The resulting CL was measured at 38.5°C in a LKB-Wallac 1251 Luminometer (LKB, Turku, Finland). Each serum was tested in duplicate.

Microscopic fluorochrome assay of phagocytosis. Bacteria were opsonized for 15 min at 37°C with 2% heat-treated bovine serum supplemented with 5% PCS. Phagocytosis was achieved as for the CL assay, except that the bacteria-to-PMN ratio was lowered to 15 by reducing the concentration of bacteria. After 30 min of incubation, cytocentrifuge smears were prepared by mixing 100 μl of the phagocytic suspension with 50 μl of PCS and 10 μl of 0.02% (wt/vol) acridine orange before centrifugation (Shandon Southern Instruments, Sewickley, Pa.). The slides were dried, and cover glasses were mounted with a conventional mounting medium (5). The slides were examined with a 100× oil immersion objective on a UV fluorescence microscope. In this assay, live organisms stained green and dead organisms stained red or yellow (5). Bacteria associated with 100 PMN were counted, and the mean numbers of ingested and killed bacteria per PMN were calculated.

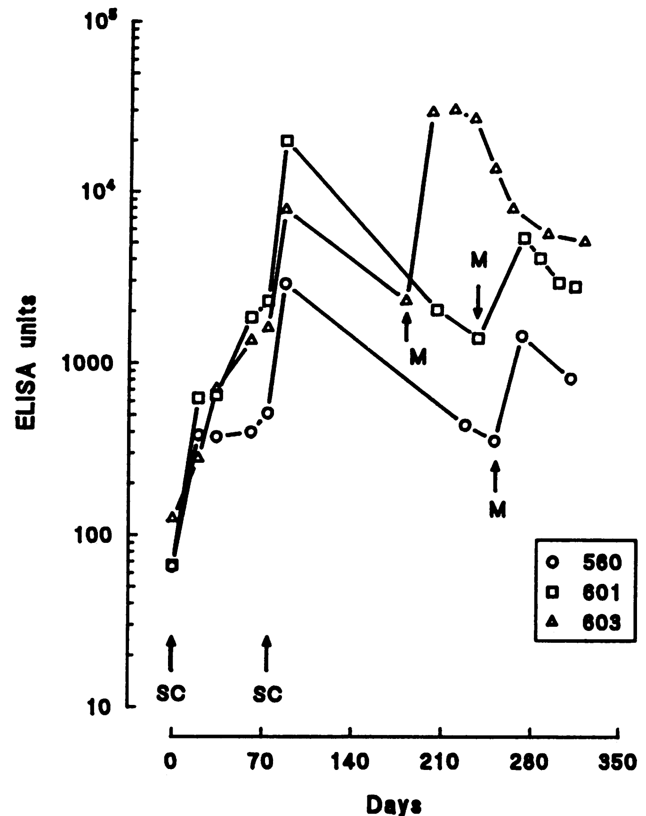

FIG. 1. Kinetics of the antibody response in sera of the three cows (560, 601, and 603) immunized with purified protein X. Sera were tested by whole-cell ELISA, using a protein X-bearing strain (443.31). SC, subcutaneous injection; M, intramammary infusion 30 days after cessation of milking.

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

RESULTS

Before immunization, all of the three cows possessed low titers of antibodies to the strains of GBS bearing protein X (Fig. 1). The first injection of protein X provoked an antibody response which peaked on day 35. The second injection gave rise to a more rapid and stronger elevation of antibody titers. The intramammary infusion acted as a booster (Fig. 1).

The specificity of the antibody response of the cows was checked with a set of 12 streptococcal strains, 4 NT/X strains, 3 R-bearing strains, and 5 strains bearing different polysaccharide-type antigens. Overall, increases in antibody titers were restricted to strains bearing protein X (Fig. 2), demonstrating that the antibody response was specific to protein X. A modest elevation of ELISA reactivity to R-bearing strains, including the R reference strain, was noted with cow 601 (Fig. 2).

The opsonic activity of bovine sera was investigated with a CL assay. Opsonization of the X reference strain (411.07) with sera of cows immunized with protein X produced PMN CL responses which exceeded those of sera obtained before

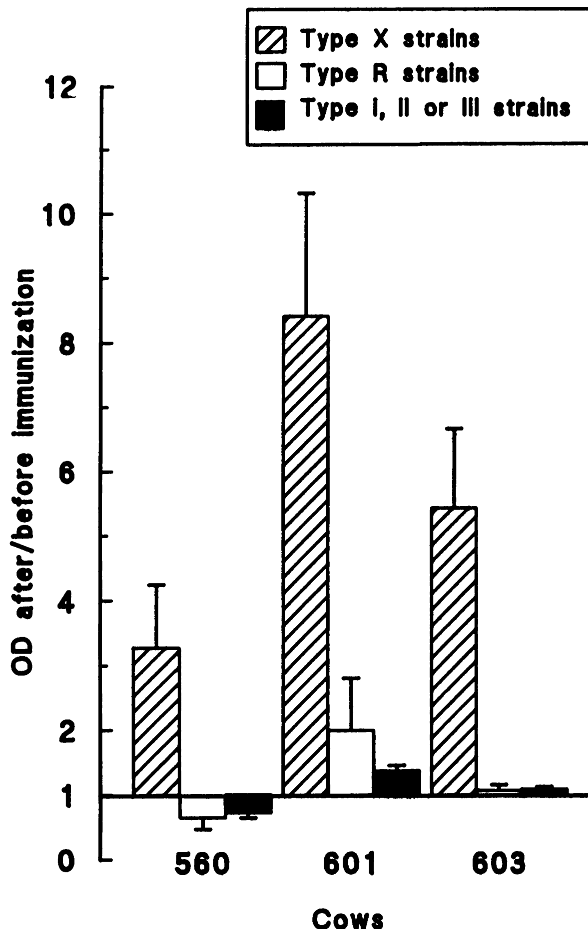

FIG. 2. Specificity of the sera from the three immunized cows. Sera were tested by whole-cell ELISA, using 12 GBS strains of different serotypes. Specificity is expressed by the ratio of the OD values of sera after immunization to the OD values of sera before immunization. Means (and standard errors of the means) are from OD values obtained with either four X-bearing strains, three R-bearing strains, or five encapsulated strains of type I, II, or III devoid of these proteins.

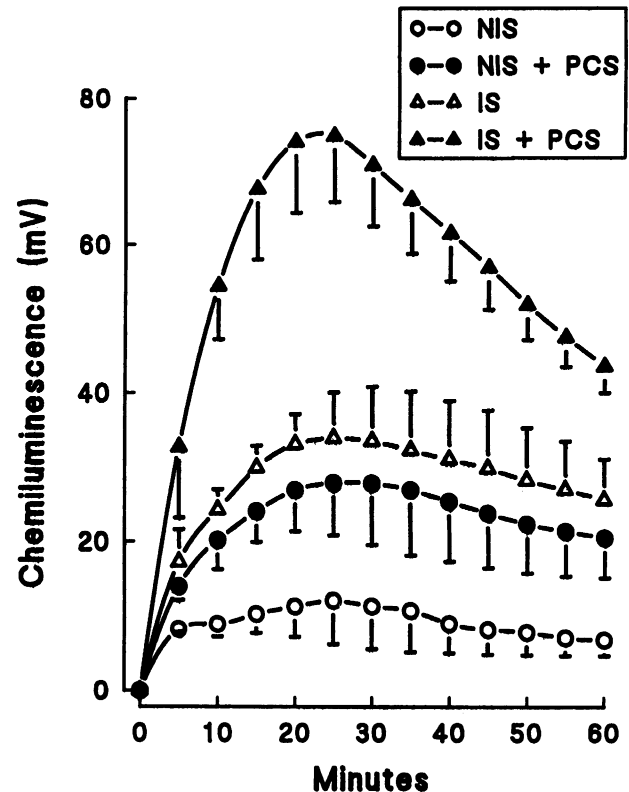

FIG. 3. PMN CL response to strain 411.07 (X reference) opsonized with either 2% preimmunization sera (NIS) or 2% postimmunization sera (IS) with or without 5% PCS as a source of complement. Values are the means (and standard errors of the means) from the sera of the three immunized cows tested in duplicate. Immune sera were taken 87 days after the first immunization with protein X.

immunization (Fig. 3). The addition of PCS to sera augmented the CL responses, suggesting that opsonization was dependent on both complement and antibodies. Similar results were obtained with strain 443.31 (results not shown).

The specificity of the opsonic activity of anti-X bovine serum was tested with a battery of seven strains that either did or did not possess protein X, opsonized with either immune (day 87 postimmunization) or nonimmune (preimmunization) sera of cow 560 (Table 2). Increases in CL responses were obtained ($P < 0.05$) after immunization with the three X-bearing strains, but not with the three encapsulated strains devoid of protein X. The PMN CL response to the R reference strain was also augmented after immunization.

Ingestion of the NT/X strains 411.07 and 443.31 opsonized with bovine serum and PCS increased after immunization ($P < 0.05$; Table 3). Ingestion of the type II reference strain was unaffected. The proportion of ingested bacteria which were killed remained unchanged (Table 3). Sera from the other two cows gave comparable results. The only difference was a larger or smaller difference in phagocytic indices after immunization with sera from cow 601 or 560, respectively.

DISCUSSION

Protein X is seldom associated with isolates responsible for human clinical infections (20). In contrast, this antigen is

TABLE 2. Peak CL responses induced by various strains of *S. agalactiae*[a]

Strain	Serotype	Peak CL response (mean ± SEM)			
		Heated serum		Heated serum + 5% PCS	
		Nonimmune	Immune	Nonimmune	Immune
411.07	NT/X	11.8 ± 3.4	51.6 ± 5.4[b]	49.6 ± 7.8	82.2 ± 9.5[b]
443.31	NT/X	10.8 ± 1.0	48.7 ± 5.4[b]	39.5 ± 6.4	100.6 ± 4.5[b]
443.21	II/X	2.4 ± 0.3	13.3 ± 0.3[b]	6.1 ± 0.5	20.1 ± 0.9[b]
411.06	NT/R	4.9 ± 0.1	11.1 ± 1.6[b]	17.7 ± 3.6	35.9 ± 9.0[b]
445.05	III/R	0.6 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	1.4 ± 0.2
411.04	II	0.9 ± 0.3	1.3 ± 0.6	2.2 ± 1.4	2.6 ± 1.8
411.05	III	1.4 ± 0.1	1.6 ± 0.1	2.8 ± 0.2	3.2 ± 0.3

[a] *S. agalactiae* sera were opsonized with 2% heated (at 56°C for 45 min) serum of cow 560 either before or after immunization (day 87) with or without complement (PCS).

[b] The difference between the values for nonimmune and immune sera is statistically significant ($P < 0.05$).

TABLE 3. Microscopic fluorochrome assay of phagocytosis by bovine PMN of three strains of *S. agalactiae*[a]

Strain (serotype)	Phagocytic index (mean ± SEM)		% Killing (mean ± SEM)	
	Nonimmune serum	Immune serum	Nonimmune serum	Immune serum
443.31 (NT/X)	1.4 ± 0.2	7.8 ± 0.9[b]	61.4 ± 5.6	65.9 ± 5.2
411.07 (NT/X)	1.4 ± 0.3	4.2 ± 0.5[b]	61.3 ± 4.4	58.3 ± 4.8
411.04 (II)	2.1 ± 0.4	2.4 ± 0.4	67.6 ± 8.8	71.8 ± 6.5

[a] Strains opsonized with nonimmune (day 0) or immune (day 87) heated serum of cow 603 plus 5% PCS. Phagocytic indices, i.e., mean numbers of ingested bacteria per PMN, and percent killing were recorded after 30 min of incubation at 38.5°C. Serum concentrations were 0.5% for X-bearing strains and 5% for the reference II strain. The values shown are from two separate experiments.

[b] The difference between mean opsonic activities of nonimmune and immune sera is statistically significant ($P < 0.05$).

frequently carried by GBS isolated from bovine mastitis (7, 10, 11), which singles it out as a potential protective antigen.

By using a purified preparation of protein X (9), we obtained in cows an immune response restricted to the strains bearing this antigen. Protein X behaved like a good immunogen. It gave rise to a secondary immune response, quicker and stronger than the first one. A further administration by the intramammary route during the dry period at a low dose (two 25-μg doses) without adjuvant, also acted as a booster.

The use of whole bacteria in the ELISA demonstrated that at least some of the antibodies induced by immunization recognized protein X in its native form. The whole-cell ELISA detected not only antibodies against protein X but also naturally occurring antibodies directed to other surface antigens. Naturally acquired antibodies against GBS occur even in the absence of known contact of cows with this bacterial species (3), and they can opsonize efficiently most of the strains (15). It is thus not surprising that preimmunization sera induced noticeable CL responses with the unencapsulated strains used in this study.

Immunization with protein X reinforced the efficiency of the opsonization of the NT/X strains. The best CL response necessitated the participation of complement. The results of the phagocytosis assay supported those obtained with the CL assay: ingestion was augmented in the presence of anti-protein X serum. At the rather low concentrations of serum used in the present study, some encapsulated strains resisted phagocytosis. The efficacy of opsonization of these strains was unaffected by immunization. In contrast, the two NT/X strains were better opsonized by the anti-protein X serum. The II/X strain used in this experiment stimulated less CL than the two NT/X strains, even when opsonized with immune serum, which means that it better resisted phagocytosis. Possibly this resulted from the presence of the type II polysaccharide capsule. Strain variation in the opsonization requirements of type II GBS, which has been reported previously (1), appears to be the result of a complex interplay of multiple surface antigens (13). Another possible contributing factor is that this II/X strain might have expressed low amounts of surface protein X and could have been consequently less affected by antibodies resulting from immunization than was strain 443.31, which produces large amounts of this protein (9).

The opsonization of the R reference strain was also improved following immunization. A serological cross-reaction between the X and R reference strains has been reported (12). One of the three cows used in the present study showed a modest elevation of antibodies to R strains, including the R reference strain. Assuming that the preparation of protein X we used in this study did not induce antibodies against another antigen of the strain 443.31, we are led to hypothesize that the R reference strain bears on its surface an antigen which has a partial identity with a determinant of protein X. This antigen could be protein R. Since four molecular species of protein R have been described (19), an epitope shared with protein X may exist on one of the R-bearing strains but not on all of them. Unfortunately, a typing for protein R specifically was not done in our study. Alternatively, the crossed immunological reaction could be due to a molecule unrelated to protein R.

Our results indicate that antibodies against protein X were opsonic, but this statement must be qualified. First, their efficiency remains to be specified by using a greater number of X-bearing strains of different serotypes. Second, opsonization with immune serum did not improve the intracellular killing of the two NT/X strains (Table 3). Overall, the number of living bacteria was reduced by a factor of 3 to 5.5, as a result of the improvement in ingestion (Table 3), which is likely to be of biological significance for the infected host. Also, CL responses were augmented by opsonization with immune versus nonimmune serum, demonstrating a stimulation of the oxidative activity of PMN. Apparently, this just reflected an increase in the number of killed bacteria, proportional to the increase in the number of ingested bacteria, but not an increase in the proportion of the ingested bacteria which were killed. The absence of further stimulation of the bactericidal activity of PMN by antibodies to protein X deserves further studies and constitutes a potential limitation to the usefulness of this protein as a protective antigen. Nevertheless, it can be noted that there was a substantial CL response with nonimmune serum, which means that phagocytes were already fairly well stimulated by bacteria opsonized with naturally acquired antibodies and complement. Indeed, the improvement in CL response with immune serum was more marked in the absence of complement than in its presence (Table 3), which supports a contribution of antibodies against protein X to the stimulation of phagocytes. The necessary further studies would be much facilitated by using affinity-purified bovine anti-X protein antibodies, which would enable us to get rid of the naturally acquired opsonins present in whole serum.

In conclusion, we have shown that the antibody response to protein X of *S. agalactiae* improved the opsonic efficiency of serum. This indicates that this protein may have more than taxonomic significance and is probably an important immunological antigen of mastitis-causing *S. agalactiae*. This prompts further studies to evaluate the opsonic activity of antibodies to protein X and the significance of the protein X for the prevention of bovine mastitis.

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