

# UNSPECIFIC BINDING OF GROUP B STREPTOCOCCAL COCYTOLYSIN (CAMP FACTOR) TO IMMUNOGLOBULINS AND ITS POSSIBLE ROLE IN PATHOGENICITY

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According to the classification scheme of Lancefield (1), strains of *Streptococcus agalactiae* were found to belong to group B streptococci (GBS).<sup>1</sup> Although Fry and Eng (2) were the first to describe three fatal group B postpartum infections and to point out the clinical importance of GBS in human infections, it was not until 1973 that GBS were firmly established as a major cause in perinatal infections (3–8). Meanwhile a wealth of information has been accumulated and has been summarized in a number of review articles (9–11).

GBS have been isolated with increasing frequency from infants with early- and late-onset septicemia and meningitis (5, 12), but they also contributed to more infrequent infections in adults such as abortion, abscesses, bacteremia, impetigo, arthritis, septicemia, and urinary tract infections (2, 5, 8, 13–20).

Besides the common group antigen, most strains of GBS share an extracellular protein first described in 1944 by Christie, Atkins, and Munch-Petersen, which accordingly was named CAMP factor (21). The CAMP factor causes lysis of red blood cells that contain at least 45 mol. % of sphingomyelin (22) and which have been exposed to *Staphylococcus aureus*  $\beta$ -toxin (sphingomyelinase). Since the designation CAMP factor neither describes the hemolytic activity nor its biological properties of Ig binding (recorded in this paper), we will use the name protein B for this extracellular substance of group B streptococci.

Used in the past to rapidly identify GBS by the CAMP reaction (23–26), protein B has been characterized as a polypeptide with  $M_r$  of 15,000 (27); 23,500 (28); 33,000 (29); and 25,000 (30). Part of the mechanism of the CAMP reaction was described by Doery et al. (31), Bernheimer et al. (28), and by work from our laboratory (32).

Besides the demonstration of mouse protective antibodies against group B streptococci (33), protein B-inactivating antibodies were also shown in human and animal sera (29), indicating that protein B is an antigen and is produced during the course of infection. However, the protein B-induced lysis of target cells in vitro remained rather an epiphenomenon and was not considered to be of relevance in vivo. Hence, the protein B has yet not been investigated in respect

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<sup>1</sup> Abbreviations used in this paper: GBS, group B streptococci; PAP, peroxidase antiperoxidase; PA, protein A; SLO, streptolysin O.

to its possible role in pathogenicity using chemically defined preparations. Thus, the work presented here provides the first evidence in a mouse infectious disease model that protein B, possibly by binding to the Fc site of a variety of mammalian Igs of the G and M classes, causes rapid septicemia with fatal outcome in mice infected with sublethal doses of GBS.

### Material and Methods

**Protein B Production.** GBS Type IIb (NCTC 8181) was obtained from Central Public Health Laboratory, London, United Kingdom. Organisms were cultured on sheep agar plates incubated at 37°C for 20–24 h in a 5% CO<sub>2</sub> atmosphere. Fermenter cultivation of group B streptococci was performed in trypticase peptone broth (No. 11921; Becton Dickinson & Co., Mountain View, CA) as reported earlier (34).

**Purification of Protein B.** Protein B was purified to homogeneity from the ammonium sulfate-precipitated culture supernatant, as previously reported (30), by ultrafiltration, hydrophobic interaction chromatography, and chromatofocusing.

**Determination of Hemolytic Activity of Protein B.** Activity was measured by the kinetic test as described (34). 1 U of protein B is defined as that amount of protein that causes change in absorbance at  $\lambda = 546$  nm of  $\Delta A = 0.1/\text{min}$  at 30°C.

**Radiolabeling of Protein B.** The method of Rosa et al. (35) was used for radioiodination. The protein B (0.4 mg; sp act,  $1.3 \times 10^5$  U/mg) was dissolved in 0.8 ml of PBS and kept at 4°C in a platinum vial required for electrolytic labeling. To this solution 20  $\mu\text{l}$  of Na<sup>125</sup>I, 2 mCi (Amersham International, Buckinghamshire, United Kingdom) were added. Electrolysis was performed at 1.8 V/20  $\mu\text{A}$  for 20 min using the equipment of Gerhard, G.m.b.H., Bonn, Federal Republic of Germany. Iodination usually resulted in a protein B preparation of 0.5–1  $\mu\text{Ci}/\mu\text{g}$  protein.

**Determination of Protein.** Protein was measured according to Peterson (36), with BSA as a standard.

**Inactivation of Hemolytic Activity of Protein B by Ig.** Hemolytic activity was determined using the kinetic assay as described (34). The interaction of Ig with protein B was detected by inhibition of the CAMP reaction after incubation with individual Ig fractions. The test was performed at 30°C in 1.5 ml cuvettes containing 1.4 ml of 0.01 M Tris-HCl, 0.145 M NaCl, 0.01 M MgCl<sub>2</sub>, pH 7.4, and 10  $\mu\text{l}$  of sphingomyelinase (E.C. 3.1.4.12.) treated SRBC. Lysis was started by the addition of 0.125  $\mu\text{g}$  of protein B in 50  $\mu\text{l}$  of buffer or with the same amount of protein B preincubated for 5 min at room temperature with 50–100  $\mu\text{g}$  of the individual Ig fractions, respectively.

**Reagents.** Human, rabbit, and bovine IgG and mouse IgM were purchased from Sigma Chemical Co., St. Louis, MO; human IgM mAbs were obtained from Behringwerke AG, Marburg, Federal Republic of Germany. Human myeloma IgM-Fc<sub>5</sub> $\mu$  fragments (chromatographically pure) were obtained from Jackson Immuno Research Laboratories, Avondale, PA. The monoclonal IgG1 and IgG2a subclasses (mouse) were a generous gift from Professor C. Sorg, Münster, Federal Republic of Germany. IgG1, clone 25F9, possessed anti-macrophage specificity; IgG2, clone 910D7, had anti-HLA-DR specificity.

**Site of Binding.** The CAMP reaction was also used to investigate the site of protein B binding to Ig. Individual IgG (100  $\mu\text{g}/20$   $\mu\text{l}$ ) and IgM (200  $\mu\text{g}/20$   $\mu\text{l}$ ) antibodies were incubated before the addition of protein B with 800  $\mu\text{g}/40$   $\mu\text{l}$  partially purified protein A (PA) (Sigma Chemical Co.) for 5 min at 20°C. Protein B (0.250  $\mu\text{g}$  in 40  $\mu\text{l}$  of 0.01 M Tris-HCl buffer, 0.145 molar in NaCl, pH 7.4) was then added to the solution, which was again incubated (5 min, 20°C) before starting lysis with 50  $\mu\text{l}$  of this mixture.

The ability of the protein B/Ig complex to recognize Fab-specific antigenic binding sites was tested in a second type of experiment. Since antibodies against streptolysin O (SLO) inhibit SLO-induced lysis of SRBC (37), this system seemed suitable to detect whether or not protein B-complexed anti-SLO antibodies retained their capacity to inhibit SLO-induced hemolysis. The kinetic assay used for hemolytic activity of protein B measurement (see above) was used, but with the modification that SRBC were not sensitized with sphingomyelinase, which constitutes an essential step in the CAMP reaction

(21, 31). SLO was purified as reported earlier (38) and kept lyophilized at  $-80^{\circ}\text{C}$  until use. SLO (3 mg/ml; SA, 2,400 HU/mg) was dissolved in 0.01 M Tris/HCl buffer, pH 7.4, containing 0.145 M NaCl and 0.01 M cystein. Complete lysis of the test erythrocytes was obtained with 50  $\mu\text{l}$  (36 HU) of SLO. This amount of SLO was neutralized by 5  $\mu\text{l}$  of an anti-SLO immune serum (rabbit) with an anti-SLO titer of 1:32.

Protein B and the anti-SLO serum were mixed at a ratio of 2:1 (vol/vol). Based on the binding ratio of protein B/IgG of 1:70, a 20-fold excess of protein B over the total amount of Ig in the antiserum was used.

**Gel Exclusion Chromatography.** Sephadex G-150 SF (Pharmacia Fine Chemicals, Uppsala, Sweden) was swollen in PBS, transferred to a C 16/40 column (Pharmacia Fine Chemicals), and equilibrated with the same buffer.  $^{125}\text{I}$ -protein B ( $\approx 40,000$  cpm/ $\mu\text{g}$ ) or  $^{125}\text{I}$ -protein B/Ig complexes prepared by incubation of  $^{125}\text{I}$ -protein B (1  $\mu\text{g}$ ) with individual Ig species (200–400  $\mu\text{g}$  in 50  $\mu\text{l}$  of PBS, for 10 min at  $20^{\circ}\text{C}$ ) were chromatographed at a flow rate of 8 ml/h at  $8^{\circ}\text{C}$ . Fractions of 2.4 ml were collected and analyzed for protein and radioactivity in the Autogamma Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). Protein values were not corrected for the small amount  $\leq 1$   $\mu\text{g}$  of  $^{125}\text{I}$ -protein B present in  $^{125}\text{I}$ -protein B/Ig complexes.

**Affinity Chromatography.** Chromatographically pure human IgM- and IgG-agarose (capacity, 1 mg protein/1 ml gel; Jackson Immuno Research Laboratories) were used for affinity chromatography. For IgM, 2 ml, and for IgG, 5 ml of gel were packed into two separate columns (C 10/20, Pharmacia Fine Chemicals), each of which was equilibrated with 300 ml of PBS. Thereafter, 50  $\mu\text{g}$  (200  $\mu\text{l}$ ) of purified protein B, trace labeled with  $^{125}\text{I}$ -protein B (100,000 cpm; specific radioactivity  $2.5 \times 10^5$  cpm/ $\mu\text{g}$ ) in PBS, were applied to each of the columns. Protein B was then adsorbed to the corresponding Ig-agarose at a flow rate of 7 ml/h at  $8^{\circ}\text{C}$ . Both the columns were thoroughly washed until no protein or radioactivity was detectable in the eluate. The protein B that bound to either the immobilized IgM or IgG was then eluted stepwise from the respective column with 50 ml (1%) of Triton X-100 in PBS and subsequently with 50 ml of 6 M guanidine-HCl in 0.1 M glycine-HCl, pH 3.0. Fractions of 2.4 ml were collected and analyzed for protein and radioactivity.

**Antigen and Immunization.** To produce antibodies, 1 mg of purified protein B (SA,  $1.3 \times 10^5$  U/mg) in 1 ml CFA (Difco Laboratories Inc., Detroit, MI) was injected subcutaneously into rabbits at multiple sites. Booster injections were given at weeks 5 and 6, and the production of antibodies was followed by the Ouchterlony technique. Blood was drawn from the animal's ear usually 6 d after the last injection.

**Streptococcal Strains.** GBS types Ia and Ib used for animal inoculation were kindly supplied by Dr. Jiri Rotta, Institute of Hygiene and Epidemiology, Prague, Czechoslovakia. The strain NCTC-8181 also used for protein B production (kindly typed by Dr. Rotta) was type IIb.

**Mice.** Male NMRI-mice, 4–6 wk old (30–33 g) used for experimental infections with GBS were obtained from the Zentrale Versuchstieranstalt, Hannover, Federal Republic of Germany.

**Mouse Passage.** GBS Types Ia, Ib, and IIb were grown in 300-ml Erlenmeyer flasks containing 100 ml of trypticase peptone broth (Becton Dickinson & Co., Mountain View, CA) with 1% yeast extract (Difco Laboratories Inc.) in a 5%  $\text{CO}_2$  atmosphere at  $37^{\circ}\text{C}$ . Cells were harvested by centrifugation (4,000 rpm,  $4^{\circ}\text{C}$ , 10 min) and washed twice in PBS. The pellet was resuspended in PBS to give an  $\text{OD}_{600}$  of 10.0, corresponding to  $2 \times 10^9$  CFU/ml. The mouse was infected intraperitoneally with 500  $\mu\text{l}$  ( $10^9$  CFU) and killed after 6–7 h (types Ia, Ib) or 14–16 h (type IIb) when the animal was moribund. Liver and spleen were removed aseptically, homogenized together in 2.5 ml of PBS, and the number of organisms was determined by spreading 0.1 ml of serial 10-fold dilutions of the homogenate on blood agar plates. The homogenate was used to infect animals in 28 repeated passages to increase the virulence of the strain (33). Finally, 10 animals were inoculated at the same time, killed, and the organs were processed as mentioned above and pooled to prepare a large volume of tissue homogenate. Aliquots of 1.0 ml of the homogenate with  $2\text{--}5 \times 10^8$  CFU were kept at  $-80^{\circ}\text{C}$  until used for animal inoculation.

TABLE I  
*Inhibition of Protein B Hemolytic Activity by Immunoglobulins*

Species	Ig class or subclass	Specificity	Inhibition
Human	IgM	Monoclonal	+
	IgG	Polyclonal	+
Mouse	IgM	Polyclonal	+
	IgG1	Monoclonal	+
	IgG2a	Monoclonal	+
Rabbit	IgG	Polyclonal	+
Bovine	IgG	Polyclonal	+

*Effect of Protein B in Experimental Infection.* The mouse infectious disease model described above was used to demonstrate the influence of protein B on experimental GBS infection. Four groups of animals (A–D) were formed. Animals of groups A–C were infected intraperitoneally with a sublethal dose of  $10^4$  CFU in 200  $\mu$ l tissue homogenate for types Ia and Ib, and with  $10^7$  CFU in 200  $\mu$ l for type IIb. Animals of group B were, in addition, injected in the tail vein with 30  $\mu$ l of sterile filtered protein B in PBS (115  $\mu$ g; SA,  $1.1 \times 10^5$  U/mg) at 0, 1.5, 3, 4.5, 6, 7.5, and 9 h after infection. The total amount of protein B was 0.8 mg. As an appropriate control, group C animals received in parallel the same volume ( $7 \times 30 \mu$ l) of PBS intravenously. Group D animals not infected received 0.8 mg of native protein B at corresponding intervals. It should be noted that type Ia and Ib were very sensitive to storage in diluted buffer as well as to freezing and thawing. Therefore, the appropriate dilutions were made immediately before use and the number of organisms injected was controlled each time.

*Detection of Protein B in Tissue Sections of Infected Mice.* The PAP method (39) was used to detect protein B in tissue sections of mice infected intraperitoneally with a lethal dose of the GBS-containing tissue homogenate ( $10^8$  CFU). The reagents, swine-anti-rabbit globulin and the PAP complex were both obtained from Dakopatts G.m.b.H., Hamburg, Federal Republic of Germany. 3-Amino-9-ethylcarbazole (4 mg in *N,N*-dimethylformamide) was used as a peroxidase substrate. Tissue sections (3–5  $\mu$ m thick) were prepared by freeze fractioning of tissue blocks of liver or spleen. Before the addition of the PAP complex, tissue sections were incubated with antibodies derived from a rabbit that had been immunized with purified protein B, as described (see above).

## Results

*Interaction of Protein B with Ig.* Besides the specific binding of antibodies to protein B (29) by antigenic determinants we observed recently that antibodies of the IgG and IgM classes inhibited the CAMP reaction in vitro although the presence of protein B-specific antibodies was unlikely. To investigate this interaction in more detail, protein B was incubated with the Ig fractions listed in Table I. It was found that all the IgG and IgM antibodies tested and derived from different mammalian species inhibited the protein B-induced lysis of SRBC. Interestingly, the CAMP reaction was also blocked by one monoclonal IgM (human) and two monoclonal IgG antibodies (mouse) lacking protein B determinant specificity. The amount of Ig needed to inhibit the CAMP reaction differed for IgG and IgM. The inhibitory molar ratio of protein B to IgM was found to be 1:20, and 1:70 for IgG.

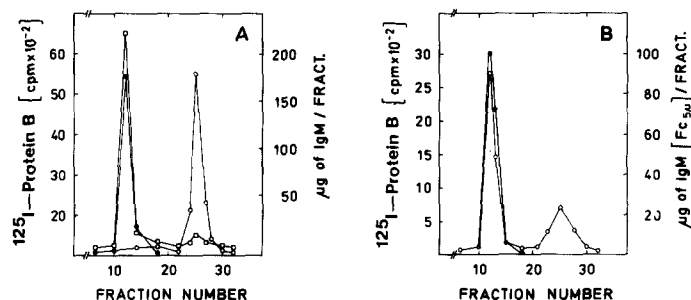


FIGURE 1. Characterization of  $^{125}\text{I}$ -labeled protein B/Ig complexes by gel exclusion chromatography on Sephadex G-150. The curves show (A) the elution behavior of  $^{125}\text{I}$ -protein B (cpm) in the absence (○) and in the presence (□) of human IgM mAbs. (●) IgM ( $\mu\text{g}$  protein per fraction) in the complex. (B) Coelution of IgM- $\text{Fc}_{5\mu}$  fragments (●;  $\mu\text{g}$  protein/fraction) with  $^{125}\text{I}$ -protein B (○; cpm) is depicted.

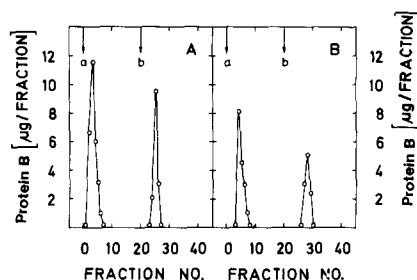


FIGURE 2. Demonstration of protein B binding to IgG and IgM agarose by affinity chromatography. (A) (○) elution of protein B ( $\mu\text{g}$  protein/fraction) from IgG agarose with (a) 1% Triton X-100 and (b) guanidine/HCl (6 M). (B) Curves show the same elution pattern after desorption of protein B from IgM agarose with Triton X-100 (a) and guanidine/HCl (b). See A.

Furthermore, the hydrodynamic properties of the protein B/Ig complexes were studied by gel exclusion chromatography on Sephadex G-150. Thus, the elution profiles of the  $^{125}\text{I}$ -protein B/IgM (human monoclonal) and  $^{125}\text{I}$ -protein B/IgM- $\text{Fc}_{5\mu}$  complexes were compared with that of  $^{125}\text{I}$ -protein B in the absence of Ig. It is obvious that both IgM (Fig. 1A) and IgM- $\text{Fc}_{5\mu}$  antibody fractions (Fig. 1B) were coeluted with the  $^{125}\text{I}$ -protein B (Fig. 1A and B) with the void volume ( $V_0 = 27$  ml), whereas  $^{125}\text{I}$ -protein B alone emerged from the column with an elution volume of  $V_e = 58$  ml (Fig. 1A). The same elution pattern was seen when complexes of protein B with the other Ig species listed in Table I were passed through the column (data not shown).

The stoichiometry of protein B/IgG or IgM complex formation and the binding forces between the components were analyzed by column chromatography with immobilized human IgG or IgM. When purified protein B was applied to the IgG-agarose column at a molar ratio of protein B/IgG of 1:25, complete adsorption was observed. Protein B was not removed by extensive washings with PBS but was desorbed to ~65% of total activity with 1% Triton X-100 in PBS (Fig. 2A; a). Elution of the residual protein B (35%) was finally achieved by applying 6 M guanidine-HCl in 0.1 M glycine, pH 3.0 (Fig. 2A; b). When IgM agarose was used, protein B was bound to IgM at a molar ratio of 1:1. Desorption

TABLE II  
*Identification of the Site of Binding of Protein B at the Immunoglobulin*

CAMP reaction	Lysis	SLO reaction	Lysis
Protein B + anti-SLO	–	SLO + anti-SLO	–
Protein B + (anti-SLO/SLO complex)*	–	SLO + (anti-SLO/protein B)	–
Protein B + (anti-SLO/PA)	+	SLO + (anti-SLO/PA)	–

\* Components in parentheses were incubated together before the addition of protein B or SLO.

with the same eluents resulted in the same elution profile as depicted in Fig. 2B. The experiments with both IgG- or IgM-agarose indicated that the amount of protein B bound to immobilized IgG or IgM differed from that found in protein B/IgG or IgM complexes in aqueous solutions.

*Site of Binding of Protein B to Ig.* The coelution of  $^{125}\text{I}$ -protein B with IgM-Fc<sub>5μ</sub> (Fig. 1B) suggests that protein B may bind in a PA-like way to the Fc sites of Ig. Since the binding of Ig to protein B can be monitored by the inhibition of the protein B-induced lysis, it seemed possible to define more precisely the site of binding of the protein B to the Ig molecule by applying the kinetic test and using substances competing either for the antigen recognition or Fc site of the Ig. The first type of interaction was investigated using an anti-streptolysin O antiserum (rabbit; anti-SLO titer, 1:32) neutralizing the hemolytic activity of SLO. When protein B was incubated with the anti-SLO serum, protein B-induced lysis was blocked (Table II). The protein B/anti-SLO complex, however, was still capable of binding to SLO, thereby inhibiting streptolysin O-induced lysis in the hemolysis test (Table II). Protein B was used in this experiment in a 20-fold excess over the precalculated amount of Ig present in the rabbit antiserum. The experiment revealed that the Fab site of anti-SLO antibodies complexed by protein B or PA retained their function to specifically bind their antigen (SLO), thus inactivating the hemolytic activity of SLO.

Additionally, PA and protein B were used to compete with each other for the binding at the Fc site of the Ig molecule or anti-SLO/SLO complex. The experiment showed that the anti-SLO/SLO complex was still able to bind protein B and to inhibit the CAMP reaction. When PA was incubated with the anti-SLO/SLO complex or one of the Ig species (listed in Table I) before the addition of protein B, complete lysis of SRBC by protein B was observed (Table II). Appropriate controls indicated that neither PA nor protein B, once fixed to the Ig molecule, was capable of displacing each other from their binding site. In addition, protein B and PA were not lytic by themselves in the SLO system (Table II).

*Protein B in Pathogenicity.* Although there was no evidence from the literature that protein B was involved in pathogenicity, the unspecific binding of protein B to IgG or IgM molecules suggested to use that protein B could play a role in GBS infections.

Our preliminary experiments revealed that mice infected intraperitoneally with a single dose of GBS, type Ia and Ib of  $10^3$  CFU, exhibited no signs of illness. Mice inoculated intraperitoneally with  $10^4$  CFU became slightly ill but recovered usually within 2 d. When killed after this time, liver and spleen were mostly sterile, whereas animals killed after 24 h still showed a colony count of

TABLE III  
*Effect of Protein B in Experimental Infection with GBS Type Ia*

Group	Mice treated with:	Number of animals	Infectious dose (CFU)	Signs of illness	CFU in liver and spleen homogenate/ml (24 h p.i.)*	CFU in blood/ml (24 h p.i.)
A	GBS	9	10 <sup>4</sup>	Slightly ill <sup>‡</sup>	0–10 <sup>5</sup>	0–10 <sup>3</sup>
B	GBS + protein B	11	10 <sup>4</sup>	Severely ill <sup>§</sup>	10 <sup>7</sup> –10 <sup>8</sup>	10 <sup>7</sup> –10 <sup>8</sup>
C	GBS + PBS	10	10 <sup>4</sup>	Slightly ill <sup>‡</sup>	0–10 <sup>5</sup>	0–10 <sup>3</sup>
D	Protein B	8	—	Healthy	Sterile	Sterile

\* p.i., postinfection.

<sup>‡</sup> Slightly ill: rough fur, weak hyperventilation, reduced activity.

<sup>§</sup> Severely ill: rough fur, strong hyperventilation, stupor, closed and/or glued eyes.

TABLE IV  
*Effect of Protein B in Experimental Infection with GBS Type Ib*

Group	Mice treated with:	Number of animals	Infectious dose (CFU)	Signs of illness	CFU in liver and spleen homogenate/ml (24 h p.i.)	CFU in blood/ml (24 h p.i.)
A	GBS	12	10 <sup>4</sup>	Slightly ill*	0–10 <sup>5</sup>	0–10 <sup>3</sup>
B	GBS + protein B	8	10 <sup>4</sup>	Severely ill <sup>‡</sup>	10 <sup>7</sup> –10 <sup>8</sup>	10 <sup>7</sup> –10 <sup>8</sup>
C	GBS + PBS	8	10 <sup>4</sup>	Slightly ill*	0–10 <sup>5</sup>	0–10 <sup>3</sup>
D	Protein B	8	—	Healthy	Sterile	Sterile

\* Slightly ill: rough fur; weak hyperventilation, reduced activity.

<sup>‡</sup> Severely ill: rough fur; strong hyperventilation; stupor; closed and/or glued eyes.

10<sup>2</sup>–10<sup>5</sup> CFU/ml in the homogenate of spleen and liver (Tables III and IV; group A). The blood of these animals was sterile at this stage of infection. However, when mice were treated over a period of 9 h with seven successive injections in the tail vein of 30  $\mu$ l each of purified protein B (total, 210  $\mu$ l = 0.8 mg) together with a sublethal dose of GBS (intraperitoneally), the animals became severely ill and occasionally died. Colony counts of the homogenate of liver and spleen of these animals after 24 h were between 10<sup>7</sup> and 10<sup>8</sup> CFU/ml, and the same number of organisms was found in 1 ml blood (Tables III and IV; group B). Mice treated with the same sequence of intravenous injections of protein B but not infected with GBS remained healthy (Tables III and IV; group D). This was also true for GBS-infected animals receiving seven repeated intravenous injections (7  $\times$  30  $\mu$ l) of PBS as a control. The blood of these animals was sterile, although colony counts in the tissue homogenate amounted to 10<sup>3</sup>–10<sup>5</sup>/ml (Tables III and IV; group C). Animals receiving a sublethal dose of GBS only (Tables III and IV; group A) showed the same colony counts and recovered usually within 2 d. Mice were also infected according to the before-mentioned protocol with GBS type IIb (NCTC 8181). In contrast to type Ia and Ib, the lethal dose was found to be 10<sup>8</sup> CFU, although the strain has been passaged 28 times. The sublethal dose was determined to be 10<sup>7</sup> CFU. The effect of protein B on mice infected with type IIb (data not shown) was the same as documented for animals infected with type Ia and Ib.

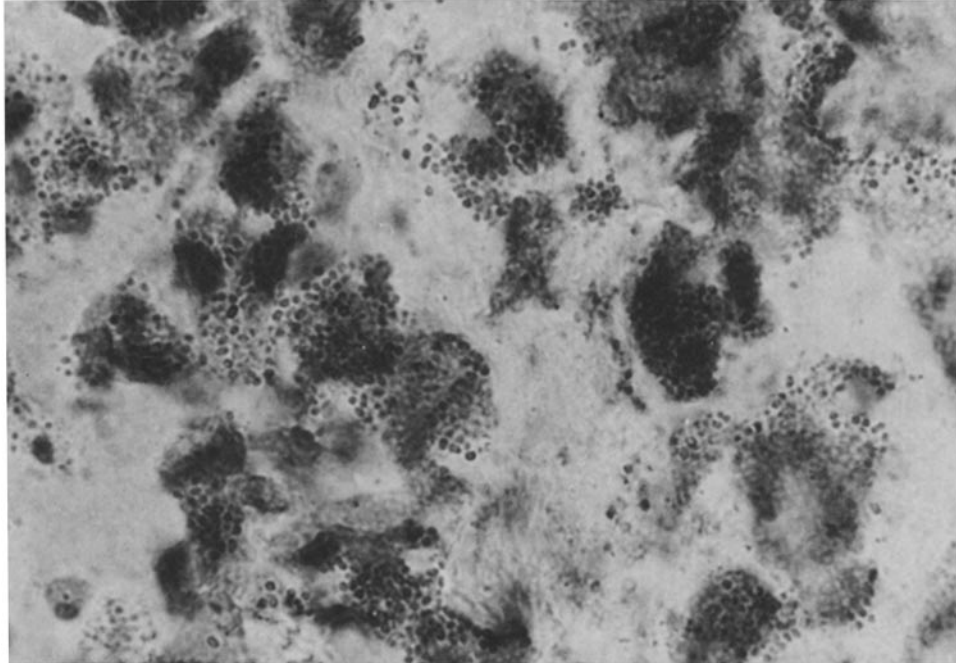


FIGURE 3. Detection of protein B in tissue sections of the liver of a mouse infected with GBS. Tissue sections (3–5  $\mu\text{m}$ ) prepared by freeze sectioning were incubated with rabbit antibodies against purified protein B and stained subsequently by the PAP method. Masses of protein B-producing cocci are visible in and outside of macrophages. Individual cocci differ greatly in their behavior to stain with peroxidase. Magnification,  $\times 1,500$ .

*Detection of Protein B in Tissue Sections.* Since the protein B used in this study was produced by fermenter cultivation in artificial medium it seemed important to also demonstrate its *in vivo* production by GBS in the infected animal, although it has been shown earlier (29) that animals responded to GBS infections by elevated antibody titers against protein B. When tissue sections after freeze sectioning were incubated with anti-protein B antibodies (rabbit) and subsequently stained using the PAP method, clusters of protein B-producing cocci were seen in and outside of macrophages. Fig. 3 shows a section of the liver of an infected mouse in which numerous macrophages with engulfed GBS are seen. The intensity of staining differs considerably for individual cocci, showing some with a strong and others with a weaker peroxidase reaction.

The same histologic pattern was seen after incubation of tissue sections with an anti-protein B antibody (rabbit) before fluorescence staining with an anti-rabbit FITC conjugate. Furthermore, fluorescence staining revealed that antigenic material was found not only on the surface of cocci but was also found as halo shapes around clusters of cocci outside of the macrophages (data not shown).

### Discussion

Surface components with the capacity to bind in a nonimmune reaction to the Fc part of the Ig have been described for *S. aureus* (protein A) and streptococci



of different serogroups (40–43). With the exception of PA, these Fc receptors on bacterial cell surfaces have not been defined in respect to their chemical nature. In contrast, the protein B of group B streptococci represents a well-defined polypeptide of known physicochemical properties (24–26, 28–32, 44) that is released from the organisms during the mid- to postlogarithmic phase of growth into the medium as a soluble extracellular protein of strongly amphiphilic properties (32, 34). In addition, we have been able to show that protein B is also produced *in vivo* during the course of experimental GBS infection (Fig. 3). The protein B that may be compared with the soluble fraction of PA can bind IgG and IgM Ig in a nonimmune reaction at the Fc site of the Ig molecules. Besides the binding of Ig of the -G and -M classes from a variety of mammalian species (Table I). Protein B was found to combine also with IgG subclasses and with both monoclonal and polyclonal IgG and -M globulins. In contrast to the properties of the Fc receptors found on bacterial cell surfaces of *S. aureus* and group A, C, G, and U streptococci (40–43), protein B was able to bind to the IgM-Fc<sub>5μ</sub> fragment of human myeloma protein (Fig. 1), thereby blocking the CAMP reaction (Table II).

The binding forces active in protein B/Ig complex formation did not differ considerably for either IgG or IgM when studied by affinity chromatography with immobilized Ig (Fig. 2). However, the dissociation behavior of the protein B/Ig complex in the presence of Triton X-100 or guanidine-HCl in affinity chromatography (Fig. 2) suggests that two different complexes with different dissociation constants exist under given conditions. Moreover, it should be mentioned that an additional type of protein B/Ig complex is formed by the interaction of protein B with specific antibodies (29), which exhibit true determinant specificities for the protein B. Preliminary experiments indicated that an immunoprecipitate is obtained in agar gel diffusion using purified protein B and antibodies raised against purified protein B in the rabbit. No such precipitates were visible when protein B was complexed in agar gel with Ig fractions lacking protein B specificity.

In fluid phase complexes the ratio of IgG or IgM to protein B was estimated to be 1:70 and 1:20, respectively. It was thus conceivable that relatively small amounts of protein B released in systemic infections could effectively impair the immune response of the host. It was found that mice infected intraperitoneally with a sublethal dose of GBS and that received in parallel seven repeated intravenous injections of purified protein B developed a severe septicemia within 24 h after infection. The presence of GBS in the blood drawn from the heart of infected animals after 24 h was documented by positive blood cultures in which  $10^8$  CFU/ml were found. Animals that developed a severe GBS infection usually harbored up to  $10^7$ – $10^8$  CFU in the combined liver and spleen tissue homogenate. When tissue sections of liver or spleen of infected animals were investigated after 15 h using the PAP method (39), large numbers of cocci were found, the majority of which were spread onto the capsular surface of the organs. Few microabscesses were found at this stage of septicemia, although numerous clusters of macrophages with phagocytized cocci were seen (Fig. 3). It was found unexpectedly that not only soluble extracellular protein B (mol wt 25,000) was stained outside of the cocci, but that the cocci themselves were stained. The intensity of

staining of individual cocci varied greatly regardless of the staining technique applied (i.e., peroxidase or FITC). It seems possible that GBS of different growth phases exhibit differences in their metabolic activities, which consequently effects the biosynthesis and/or release of protein B *in vivo*.

Furthermore, the immunohistological findings suggest that protein B is not only present extracellularly but also on the surface of GBS, thus exhibiting Ig/Fc receptor function. The number of receptors exposed on the cell surface may, however, depend on the growth phase of individual cocci, as stated above. This aspect could be of relevance in discussing the controversial results on the binding of IgG to group B or D streptococci (40, 41).

Although our data provide strong evidence that protein B is involved in pathogenicity of GBS infections, care must be taken in extending this view to infectious disease models other than mouse. Presently, we are far from an understanding of the sequence of events initiated by the interaction of protein B with Ig or other factors of the immune system, such as complement (42) or phagocytosis (45). Further work will be needed to disclose the role of protein B and the fate of the protein B/Ig complexes, especially in human infections (i.e., early- and late-onset septicemia in neonates). This work could profit from the data presented here on the hitherto unknown properties of protein B.

### Summary

The protein B of group B streptococci can bind in a nonimmune reaction to Ig of the IgG and IgM classes of various mammalian species (i.e., human, mouse, rabbit, and bovine). Protein B binding involves the Fc parts of both IgG and IgM molecules. Monoclonal or polyclonal IgG or IgM and the IgM-Fc<sub>5μ</sub> fragment of human myeloma protein combined with the protein B thereby inhibiting protein B-induced hemolysis in the CAMP reaction. The protein B/Ig complex can be dissociated with 1% Triton or guanidine-HCl (6 M). Mice infected intraperitoneally with sublethal doses of group B streptococci (GBS) and that received seven repeated intravenous injections of highly purified protein B during the first 9 h of infection developed fatal septicemia within 24 h with colony counts of up to 10<sup>8</sup> CFU/ml in the blood. Animals treated in the same way with either PBS or trypsinized protein B recovered. The protein B itself was not pathogenic when injected into healthy mice. Tissue sections of liver or spleen from mice infected with a lethal dose of GBS revealed the presence of protein B together with large numbers of cocci when stained by the peroxidase method using specific antibodies raised against purified protein B in the rabbit.

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