

# FIBRONECTIN ENHANCES THE OPSONIC AND PROTECTIVE ACTIVITY OF MONOCLONAL AND POLYCLONAL ANTIBODY AGAINST GROUP B STREPTOCOCCI

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Fibronectin (FN)<sup>1</sup> is a high molecular weight glycoprotein found in the plasma and intracellular matrix which participates in reticuloendothelial clearance of particles and helps to maintain vascular stability (1). Recently, FN has been shown to promote the attachment of immunoglobulin-coated erythrocytes to human mononuclear phagocytes (2). This effect has been postulated (3) to be due to an effect of FN on the number or location of Ig Fc receptors on the surface of the phagocytic cells. In the present studies, we have examined the interaction between FN, monoclonal and polyclonal antibody, and a major bacterial pathogen, group B streptococci, in both opsonic and protective assays. The data suggest that this high molecular weight glycoprotein has an additive or even synergistic effect with these Ig preparations in promoting both bacterial uptake and protection.

## Materials and Methods

*Preparation of Organisms.* Human isolates of group B streptococci were cultured in Todd Hewitt broth (Difco Laboratories Inc., Detroit, MI) at 37°C for 16 h. The organisms were washed three times in sterile phosphate-buffered saline (PBS) (4,500 ml distilled water, 5.2 g Na<sub>2</sub>HPO<sub>4</sub>, 0.9 g KCl, 0.9 g KH<sub>2</sub>PO<sub>4</sub>, 36 g NaCl) and adjusted to an optical density of 0.9 at a wavelength of 620 nm (Spectronic 20; Bausch & Lomb Inc., Rochester, NY). These standard suspensions contained ~5.0 × 10<sup>8</sup> colony-forming units per milliliter. Organisms were also grown overnight in the presence of tritiated thymidine (5 μCi/ml) for use in the radiolabeled bacterial uptake experiments.

*Preparation of Polymorphonuclear Leukocytes.* Human polymorphonuclear leukocyte (PMN) suspensions were prepared from heparinized (10 U/ml) whole blood. After sedimentation of the erythrocytes, the leukocyte-rich plasma was removed and centrifuged and the leukocyte button was washed twice in PBS. The cells were then resuspended in a final concentration of 1 × 10<sup>7</sup> PMN/ml.

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<sup>1</sup> *Abbreviations used in this paper:* CL, chemiluminescence; FN, fibronectin; IgIV, immunoglobulin modified for intravenous use; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; SE, sheep erythrocyte.

*Opsonization Procedure.* Opsonization of organisms was carried out at 37°C with rotation in the presence of a human polyclonal Ig modified for intravenous use (IgIV) (Cutter Laboratories, Inc.) or murine monoclonal antibody prepared as described below.

*Preparation of the FN.* FN was prepared at Cutter Biologicals by modifications of previously described procedures (4). Cryoprecipitate was removed from fresh frozen plasma and solubilized in distilled water at 20°C. Acidification to pH 6.5 was carried out and then the solution was chilled to 5°C. The chilled precipitate was then solubilized in PBS and contacted with gelatin-coated Sepharose (3, 4). Unabsorbed proteins were removed with 1 M urea and the FN subsequently eluted with 4 M urea. Dialysis, ultrafiltration, and pasteurization at 60°C for 10 h were then carried out. Immunoelectrophoretic analysis of the preparation revealed no contamination with gamma globulin or complement and a single precipitin arc with anti-FN and anti-whole human serum antibody (Cappel Laboratories, West Chester, PA).

*IgIV.* IgIV was obtained from Cutter Biologicals and was prepared from Cohn fraction II of human plasma by previously described methods (5). The IgIV contains antibody against the type-specific antigen of type III group B streptococci (6).

*Murine Type-specific Monoclonal Antibodies.* Murine monoclonal antibodies of IgM (6), IgG, and IgA (7) isotype were prepared as previously described. Briefly, spleen cells from mice immunized with whole type III group B streptococci were fused with either murine myeloma SP 2/0 myeloma cells (IgM) or Ag 8.653 cells (IgG and IgA) using polyethylene glycol (PEG) (Sigma Chemical Co., St. Louis, MO). The cells were then cultured in media containing hypoxanthine, aminopterin, and thymidine (HAT). Supernatant fluid was screened for type-specific antibody using enzyme-linked immunosorbent assay (ELISA) methods previously described (6, 7). Positive cultures were cloned by limiting dilution in media without aminopterin. Isotypes of the clones were determined by double diffusion in 1% agarose against isotype-specific goat anti-mouse Ig (Cappel Laboratories, West Chester, PA and Litton Bionetics Inc., Kensington, MD). Cells were frozen for future use in liquid nitrogen. To produce high-titered antibody,  $\sim 5 \times 10^6$  of the fused cells were injected intraperitoneally into BALB/c mice that had received 0.3 ml of tetramethylpentadecane 2–8 wk previously.

*Opsonic Assays.* Three assays of opsonic activity were used including a chemiluminescence (CL) procedure (8), one dependent on the use of radiolabeled bacteria (9), and light and electron microscopy. In the CL assay, 0.5 ml of the bacterial suspension containing  $\sim 5 \times 10^8$  organisms was added to 0.5 ml of the cell suspension containing  $5 \times 10^6$  PMN in  $10^{-6}$  M luminol (Sigma Chemical Co.). The mixtures were immediately placed in a Beckman LS 8000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA) and counted at 10–15-min intervals for up to 180 min. CL was expressed in cpm over time or as the area under the resulting curves (integrals) as calculated using Simpson's approximation.

Radiolabeled bacterial uptake experiments were carried out using PMN monolayers (>95% PMN) prepared on  $18 \times 18$ -mm glass coverslips as previously described (9). Bacteria (0.3 ml containing  $5 \times 10^8$  organisms) were added to the monolayers and incubated for 30, 60, or 120 min at 37°C. The coverslips were then vigorously washed to remove all attached but uningested microorganisms (as determined by light and electron microscopy) and placed into Poly Q scintillation vials (Beckman Instruments, Inc.) containing Aquasol (New England Nuclear, Boston, MA). The preparations were then counted in a scintillation counter and the percentage of ingested organisms determined. Giemsa-stained smears of coverslips were also examined by light microscopy in an attempt to differentiate ingested from attached organisms. Selected phagocytic mixtures were fixed and examined by electron microscopy using standard fixing and staining techniques (10, 11).

*Determination of Fc and C3b Receptors on PMN.* IgG- and IgM-coated sheep erythrocytes (SE) were prepared as described by Bianco (12). IgM-C3b-coated cells were prepared as described by Newman, Musson, and Henson (13) using whole human complement that had been absorbed with SE and zymosan. A 2% solution of the antibody-coated SE was incubated with an equal volume of a PMN solution containing  $1 \times 10^7$  PMN for a total

of 30 min after centrifugation. The percentage of PMN forming rosettes with three or more SE and the average number of SE attached to each PMN were then determined.

*Neonatal Rat Model.* The animal model used to determine the protective efficacy of the various preparations consisted of outbred Sprague-Dawley rats that were infected at <24 h of age as described previously (6). Briefly,  $\sim 5 \times 10^6$  type III group B streptococci in a 10- $\mu$ l vol were injected intraperitoneally. Animals also received a separate intraperitoneal injection (20  $\mu$ l) of the antibody and/or the FN. Animals were observed for mortality for at least 1 wk.

*Statistical Analysis.* The significance of differences between survival values in the animal experiments was determined by the chi-square test with Yates correction.

### Results

FN had an effect to markedly enhance the uptake of antibody-coated group B streptococci. As shown in Fig. 1, type III group B streptococci opsonized in the human IgIV promoted a significant, early peak in PMN CL generation ( $180 \times 10^3$  cpm). In contrast, FN and group B streptococci elicited only a modest increase in PMN light production after 100 min. When FN was added to a mixture of PMN and group B streptococci that were preopsonized in IgIV, a marked enhancement in CL generation was observed. The effect of FN to

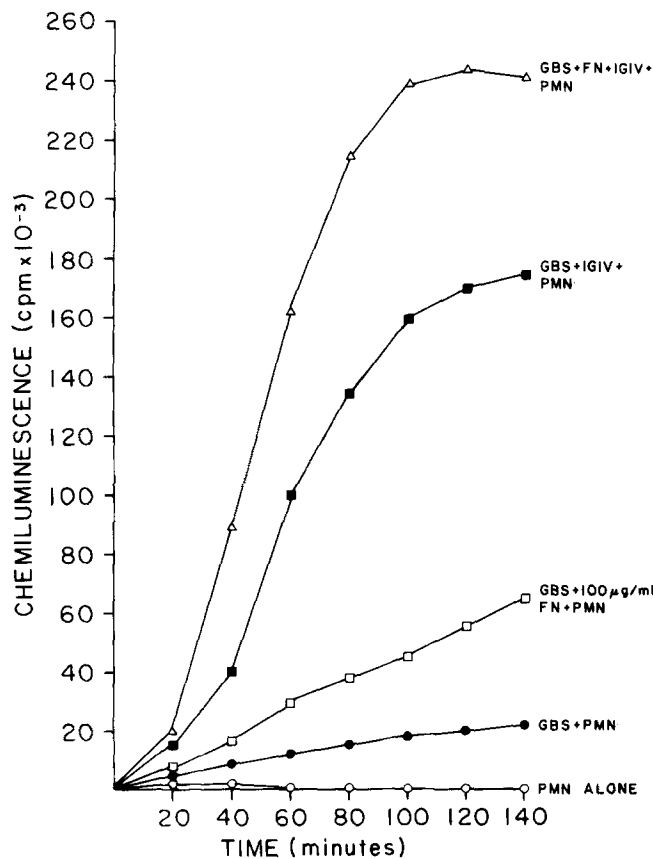


FIGURE 1. Opsonic activity of FN and IgIV for type III group B streptococci (GBS) as measured by PMN CL.

enhance CL generation by PMN exposed to IgIV-coated streptococci was somewhat dose dependent, with the maximum effect occurring at physiologic concentrations from 100 to 300  $\mu\text{g}/\text{ml}$  (data not shown). Experiments were next carried out to determine if FN was acting on the organisms or on the PMN in the CL assay. The addition of FN to the preopsonization mixture containing group B streptococci and IgIV, followed by washing before the addition of PMN, had the effect of mildly enhancing CL generation (data not shown). In contrast, preincubation of PMN suspensions with the FN followed by washing markedly increased the response to IgIV-coated streptococci (Fig. 2). The most prominent response always occurred, however, when the FN was present in the final reaction mixture (Fig. 1).

To verify the results of the CL assay of opsonization, we used a radiolabeled bacterial uptake technique (9). In three separate experiments, IgIV promoted the uptake of an average of  $36.7 \pm 6.4\%$  of the radiolabeled type III group B streptococci by PMN monolayers. FN alone, in contrast, did not promote significant uptake of the organisms by the PMN ( $1.3 \pm 2.3\%$ ). When organisms were preopsonized in IgIV and then exposed to PMN monolayers to which FN had been added, an average of  $56.3 \pm 12.7\%$  of the bacteria were phagocytized.

We next used rosetting techniques to determine if FN had an effect on Ig or complement receptors on human PMN. Preincubation of PMN with FN (100–300  $\mu\text{g}/\text{ml}$ ) for 1 h slightly decreased the percentage of cells forming rosettes with IgG-coated SE ( $74.1 \pm 6.4\%$  untreated to  $56.1 \pm 8.1\%$  with FN). Essentially no effect was observed on the expression of Fc receptors for IgM ( $0\text{--}3.0 \pm 5.2\%$ ) or C3b receptors ( $48.7 \pm 16.0$  to  $51.2 \pm 15.8\%$ ), however. There was also no effect of FN to increase the average number of SE attached to each PMN with any of the indicator cells.

We next examined the opsonic effects of FN in combination with the three type-specific monoclonal antibody preparations. As can be seen in Table I, the

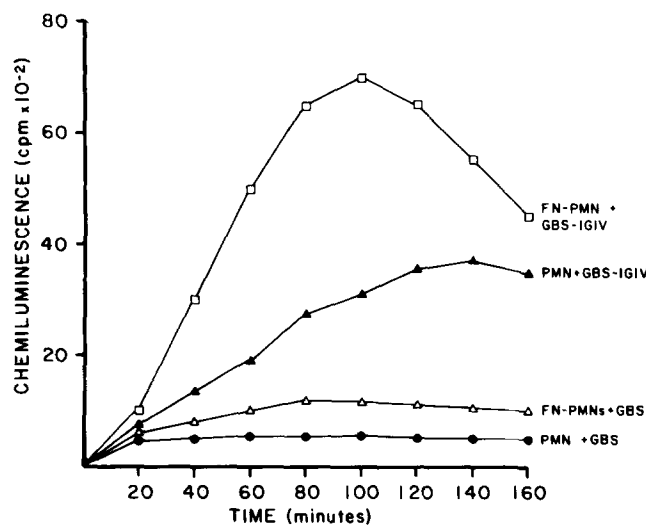


FIGURE 2. Effect of FN preincubation with PMN (FN-PMN) on CL induced by IgIV-opsonized group B streptococci.

TABLE I  
*Effect of FN on the Opsonic Activity of Monoclonal Type-specific Antibody Against Type III Group B Streptococci as Measured by PMN CL*

Opsonic mixture	Peak CL (cpm $\times 10^3$ )	Integral* (cpm $\times 10^3$ )
Media alone	1.0	88
FN alone	0.4	54
IgG	40.1	5,741
IgG + FN	118.4	11,498
IgM	10.0	1,367
IgM + FN	54.4	6,034
IgA	39.5	6,046
IgA + FN	168.6	22,253

Mixtures contained  $5 \times 10^8$  organisms and  $5 \times 10^6$  PMN in  $10^{-6}$  M luminol.

\* Calculated area under the CL curve using Simpson's approximation. FN, 100  $\mu$ g/ml; IgG, 90  $\mu$ g/ml; IgA, 90  $\mu$ g/ml; IgM, 9  $\mu$ g/ml.

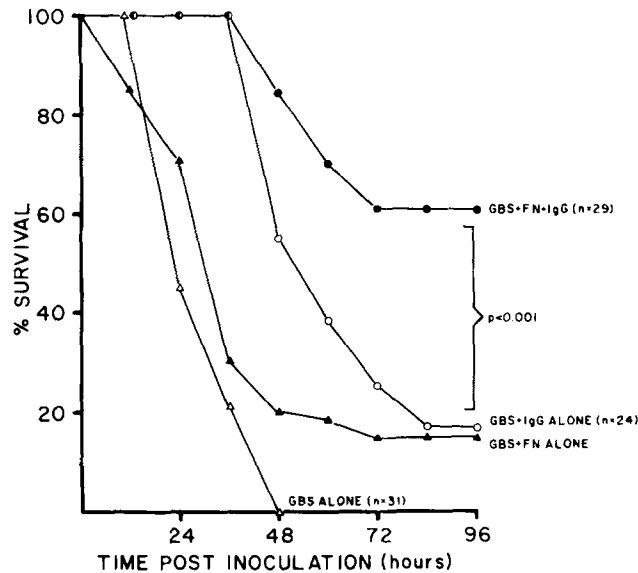


FIGURE 3. Effect of FN and monoclonal IgG type-specific antibody administration on the survival of neonatal rats infected with type III group B streptococci (GBS). Rats received  $5 \times 10^6$  streptococci, 1.2 mg/kg of IgG monoclonal antibody, and/or 5 mg/kg of FN.

IgG, IgM, and IgA monoclonal antibody preparations produced at least some increase in PMN CL generation when used alone to opsonize group B streptococci. In contrast, the FN alone failed to produce a significant increase in CL. The addition of FN to the final reaction mixture in a concentration of 100  $\mu$ g/ml, however, resulted in significant enhancement of CL production with each of the monoclonal preparations. Thus, FN appeared to enhance the interaction between PMN and bacteria opsonized with monoclonal IgG, IgM, and, surprisingly, IgA.

We next turned to *in vivo* protection studies to assess the biologic significance of our findings. As shown in Fig. 3, the administration of FN alone (5 mg/kg, equivalent to that reported to enhance survival in traumatized, septic humans) to neonatal rats infected with type III group B streptococci had no significant effect on survival (15%). Similarly, the monoclonal IgG preparation (1.2 mg/kg) alone offered little protection (15%). When both preparations were administered together, however, significant protection (60%) was observed ( $P < 0.0001$ ). FN also enhanced the protective efficacy of IgIV (data not shown).

Because of the marked protective efficacy of the IgM monoclonal, it was necessary to decrease the dose of this preparation to 0.004 mg/kg to study the effects of combined administration with FN. As shown in Fig. 4, the combination of the IgM monoclonal and FN also offered significantly enhanced protection against type III group B streptococcal infection (78% survival vs. ~20%;  $P < 0.001$ ). Monoclonal antibody preparations directed against the group B carbohydrate or a nonstreptococcal antigen (TGAL) had no effect on survival when administered alone or with FN.

We were unable to demonstrate significant protection by the IgA monoclonal preparation alone in our neonatal rat model (Fig. 5). FN alone failed to provide statistically significant protection in these experiments. When both preparations were administered together, however, the protection observed was  $>75\%$  ( $P < 0.001$ ). Thus, the unexpected results obtained in the CL assay, in which IgA and FN in combination appeared to promote the interaction of group B streptococci with PMN, have a corollary in this *in vivo* system.

Electron microscopy studies clearly showed that the combination of FN and

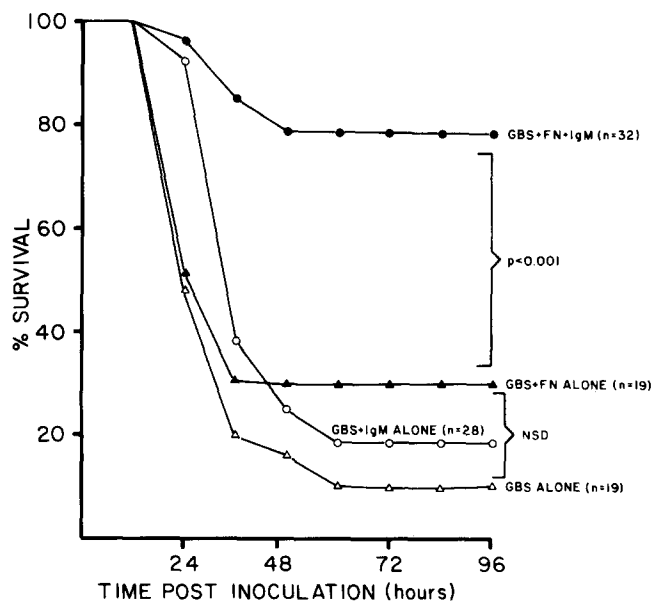


FIGURE 4. Effect of FN and monoclonal IgM type-specific antibody administration on the survival of neonatal rats infected with type III group B streptococci (GBS). Rats received  $5 \times 10^6$  streptococci, 0.004 mg/kg of IgM monoclonal antibody, and/or 5 mg/kg of FN.

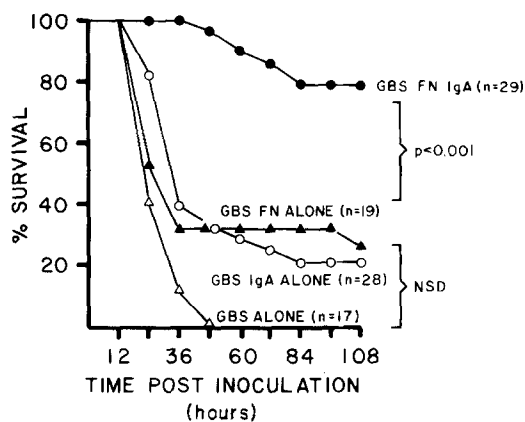


FIGURE 5. Effect of FN and monoclonal IgA type-specific antibody administration on the survival of neonatal rats infected with type III group B streptococci (GBS). Rats received  $5 \times 10^6$  streptococci, 1.2 mg/kg of IgA monoclonal antibody, and/or 5 mg/kg of FN.

Ig promoted the ingestion of streptococci by human PMN. In contrast, we were unable to demonstrate that FN alone promoted uptake of these organisms; rather it appeared that the organisms exposed to PMN in the presence of FN alone were attached to the surface rather than actually being ingested (Fig. 6).

### Discussion

The present studies indicate that FN enhances the interaction between group B streptococci, polyclonal and monoclonal antibody, and phagocytic cells important in host defense against these organisms. FN has been reported (14) to bind to *Staphylococcus aureus* and to promote attachment but not ingestion by phagocytic cells. Myhre and Kuusela (15) have shown that FN also binds to group A, C, and G streptococci; group B strains were not studied. Simpson and Beachey (16) have indicated that FN serves as the surface receptor for group A streptococci on oral epithelial cells. Thus, it is clear that FN plays at least some role in the host defense mechanism against gram-positive organisms, and particularly streptococci. The present studies are the first to show that FN promotes the interaction of group B streptococci with PMN and polyclonal or monoclonal antibody.

Bevilacqua and associates (2) reported that FN enhances the expression of Fc and C3b receptors on the surface of human monocytes. In contrast, Pommier et al. (3) could not demonstrate an effect of FN on the expression of Fc receptors on monocytes, results which are similar to ours using PMN. They pointed out, however, that receptor number does not appear to correlate with rosette formation. In results similar to ours using group B streptococci, they found that preincubation of monocytes with FN increased their phagocytic ability for C3b-, IgG-, and IgM-coated SE. In addition, we found that the FN also had the effect of promoting the interaction of group B streptococci with human PMN when combined with a type-specific IgA monoclonal preparation. IgA is not known to promote opsonization by itself, or in the presence of complement,

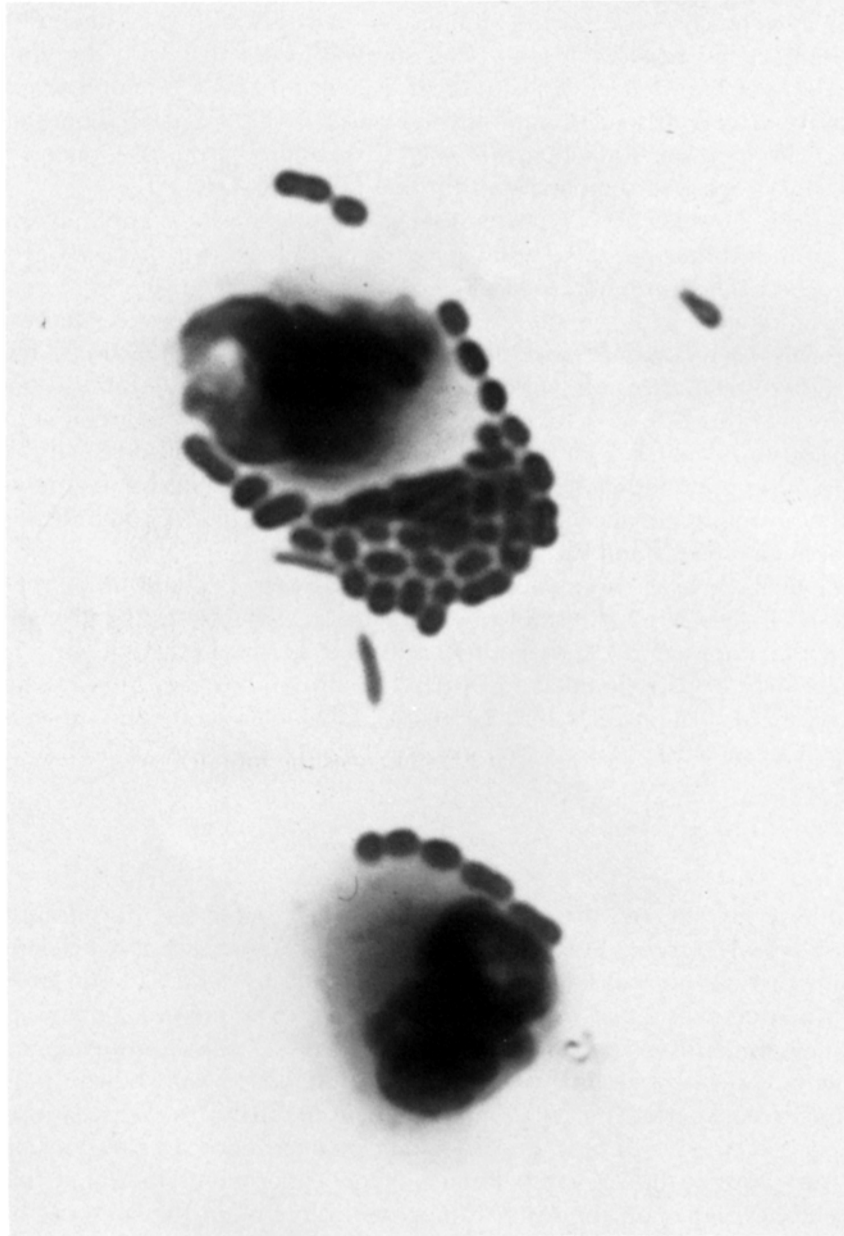


FIGURE 6. Light micrograph of human PMN exposed to unopsonized type III group B streptococci in the presence of FN.

under most circumstances. The one exception to this was the report by Lowell et al. (17) demonstrating IgA-mediated phagocytosis of *Neisseria meningitidis*.

We next turned to a neonatal rat model to ascertain the biological significance of the data we had developed with FN and the various antibody preparations. Administration of FN alone did not protect the rats from infection with type III



group B streptococci. Combined administration of FN with IgIV or the IgG or IgM monoclonal resulted in enhanced survival above that with the antibody alone. To our knowledge, this is the first evidence that FN enhances the actual protective activity of specific antibody *in vivo*. It should be pointed out that FN had no effect on survival when combined with an anti-group B monoclonal or with a nonstreptococcal monoclonal antibody (anti-TGAL).

Egan and co-workers (7) reported that the IgA monoclonal antibody that we used protected mucin-treated adult mice from challenge with type III group B streptococci. The IgA monoclonal alone, in contrast, did not protect our neonatal rats from type III group B streptococcal infection. When FN was administered along with the IgA monoclonal, however, protection was >75%. It is likely that adult mice used in the studies of Egan et al. (7) had normal FN levels. In contrast, we (18) and other (19) have found that plasma FN concentrations are significantly decreased in neonates. This could explain the differences observed in the two studies using the same monoclonal preparation. The striking finding, however, was the profound enhancement of protection induced by the administration of a combination of FN and the IgA monoclonal antibody.

Although the exact mechanism of action of FN in this and other reported studies (1-3, 14-16) is not known, it is clear that this interesting glycoprotein affects the interaction of phagocytic cells and Ig, resulting not only in the enhancement of particle uptake *in vitro*, but also in improved survival after *in vivo* bacterial challenge. It may be that optimal immunotherapy of neonatal group B streptococcal disease will involve the administration of both antibody and FN.

### Summary

We have investigated the opsonic and protective effects of fibronectin (FN) against type III group B streptococci. When used by itself, the FN failed to promote actual internalization of group B organisms. The addition of FN to group B streptococci that had been preopsonized in an immunoglobulin preparation modified for intravenous use (IgIV) or a type-specific, murine monoclonal antibody of IgG isotype markedly enhanced interaction with human polymorphonuclear leukocytes (PMN). A similar enhanced effect was observed when the FN was combined with type-specific monoclonal antibody preparations of IgM and, surprisingly, IgA isotype. Preincubation experiments indicated that the major effect was upon the PMN rather than directly on the bacteria, but we could not demonstrate an effect of FN on cell surface receptors for the Fc fragment of Ig or C3b using rosetting techniques. In addition to enhancing the *in vitro* opsonic activity of Ig, the FN significantly increased the protective effect of the polyclonal and monoclonal Ig preparations in an animal model of neonatal group B streptococcal disease. Thus, FN appears to have a critical role in the host defense mechanisms against group B streptococci.

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