Pathogenesis of *Campylobacter fetus* Infections

Failure of Encapsulated *Campylobacter fetus* to Bind C3b Explains Serum and Phagocytosis Resistance

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Abstract

***Campylobacter fetus* ssp. *fetus* strains causing systemic infections in humans are highly resistant to normal and immune serum, which is due to the presence of high molecular weight (100,000, 127,000, or 149,000) surface (S-layer) proteins. Using serum-resistant parental strains (82-40 LP and 23D) containing the 100,000-mol wt protein and serum-sensitive mutants (82-40 HP and 23B) differing only in that they lack the 100,000-mol wt protein capsule, we examined complement binding and activation, and opsono-phagocytosis by polymorphonuclear leukocytes. C3 consumption was similar for all four strains but C3 was not efficiently bound to 82-40 LP or 23D even in the presence of immune serum, and the small amount of C3 bound was predominently the hemolytically inactive iC3b fragment. Consumption and binding of C5 and C9 was significantly greater for the unencapsulated than the encapsulated strains. Opsonization of 82-40 HP with heat-inactivated normal human serum caused > 99% killing by human PMN. Similar opsonization of 82-40 LP showed no kill, but use of immune serum restored killing. Findings in a PMN chemiluminescence assay showed parallel results. Association of ^{32}P-labeled 82-40 HP with PMN in the presence of HINHS was 19-fold that for the 82-40 LP, and electron microscopy illustrated that the difference was in uptake rather than in binding. These results indicate that presence of the 100,000-mol wt protein capsule on the surface of *C. fetus* leads to impaired C3b binding, thus explaining serum resistance and defective opsonization in NHS, mechanisms that explain the capacity of this enteric organism to cause systemic infections.**

Introduction

Campylobacter fetus ssp. *fetus* is an enteric pathogen of humans that chiefly causes bacteremia and other systemic infections especially in immunocompromised hosts (1–3). The likelihood of bacteremia in *C. fetus* infection is increased nearly 1,000-fold over that due to the closely related, more common enteric pathogen, *Campylobacter jejuni* (4, 5). In previous work we have found that *C. fetus* isolates from humans are resistant to the bactericidal activity present in normal human or rabbit serum, that this resistance usually is high grade, and cannot be overcome by immune serum (6). Serum resistance is strongly associated with the presence of high molecular weight surface proteins on *C. fetus* strains (7). Recently, we identified two different laboratory-passaged strains of *C. fetus* that had lost their serum resistance. These strains, 82-40 HP and 23-B, lacked a 100,000-mol wt surface (S-layer) protein but were otherwise identical to the parent strains (82-40 LP and 23D) (7). Strain 23D but not 23B had an S-layer microcapsule identified by electron microscopy; this microcapsule was composed of a 100,000-mol wt protein (8, 9).

In the present investigation, we have used our paired strains (82-40 LP and 82-40 HP, 23D and 23B) to help elucidate the molecular basis for the serum resistance of encapsulated *C. fetus*. However, serum resistance, while probably an important virulence factor for *C. fetus*, and probably necessary for extraintestinal spread, may not be sufficient to explain disease (5). Since many of the persons affected are immunocompromised or have white blood cell dysfunction (2, 3, 7, 10), we also examined the uptake and killing of our paired *C. fetus* strains by human PMN. In the studies we report, we found virtually no binding of C3b to the encapsulated *C. fetus* strains, a phenomenon that explains the observed serum resistance and resistance to phagocytosis except in the presence of immune serum.

Methods

Media. Media included Medium 199 with Hank's balanced salt solution (HBSS, pH 7.4, Gibco, Grand Island, NY); HBSS with 1% gelatin (Hank's gel); trypticase soy agar with 5% sheep blood (BAP,[1] Pasco Laboratories, Wheat Ridge, CO); phosphate-buffered saline (PBS, pH 7.4).

Bacteria. All human *Campylobacter* isolates were from the Denver *Campylobacter* laboratory culture collection as previously described (6, 7). Typical serum-resistant *C. fetus* strains and their laboratory-derived mutants were studied (Table I). Bacteria had been frozen at −70°C and had six or fewer passages on artificial media before use in these studies. Strains were cultured on typticase soy agar with 5% sheep erythrocytes (BAP), and incubated for 24–48 h at 37°C for *C. fetus* in a microaerobic atmosphere as previously described (6). *C. jejuni* strains were incubated at 42°C but otherwise treated as above. SDS-PAGE of solubilized whole cells was performed as previously described (11). *Staphylococcus aureus* 502A and a serum-sensitive *Escherichia coli* K12 strain were used as controls for the complement-consumption and phagocytosis experiments.

Serum. Pooled normal human serum (NHS) was collected from three to five normal volunteers without any known history of *C. fetus* infection. Rabbits were immunized with encapsulated or unencapsulated *C. fetus* strains as previously described (12). Specificities of the antisera were defined by immunoblot and radioimmunoprecipitation as previously described (7, 13). Titers of the immune rabbit sera to homologous organisms in an ELISA (14) to detect IgG to *C. fetus*

Received for publication 13 April 1987 and in revised form 12 November 1987.

The Journal of Clinical Investigation, Inc.
Volume 81, May 1988, 1434–1444

1. *Abbreviations used in this paper:* BAP, trypticase soy agar with 5% sheep blood plate; HINHS, heated-inactived normal human serum; NHS, normal human serum; SDW, sterile distilled water.

Table I. Surface Proteins and Serum Resistance of Campylobacter Species

Strain	Source	Log_{10} kill in standard assay*	LPS serotype‡	Presence of 100k surface protein§
C. fetus				
82-40 LP	Human blood	<0.01	A	+
82-40 HP	Laboratory derivative of 82-40 LP	1.02	A	−
23D (84-32)	Bovine fetus‖	<0.01	A	+
23B (84-54)	Laboratory derivative of 23D‖	1.74	A	−
81-170	Bovine vagina	1.47	B	−
C. jejuni				
79-193	Human feces	3.76	C	−
81-93	Human feces	1.83	C	−

* Serial dilutions of isolates from 10^3 to 10^5 organisms/ml were incubated for 60 min at 37°C in 10% PNHS. Quantitative bacterial cultures were done and log_{10} kill was calculated as described in *Methods*. Results are means±SE for 2–12 determinations. ‡ Serotyping performed as previously described (11). § By SDS-PAGE and radioiodination as described (6). ‖ As described in references 8 and 9.

always were greater than 1:3,200. Aliquots of NHS, normal rabbit serum, or immune rabbit serum were heated at 56°C for 30 min to block complement activation. For use in some experiments, 2.0 ml of immune rabbit serum to 82-40 LP were absorbed six times with ~ 10^{10} 82-40 LP CFU for 60 min at 37°C, and the bacteria were removed by centrifugation at 12,000 *g* for 10 min. All sera were stored at −70°C until used in the assays.

Serum bactericidal assay. The susceptibility of the *C. fetus* and *C. jejuni* strains to the bactericidal activity present in NHS was assessed in a standardized assay, as previously described (6). In brief, 24-h cell cultures were diluted in Medium 199 with HBSS to concentrations of 10^3 to 10^5 CFU/ml, then incubated for 60 min at 37°C with 10% pooled serum from healthy adults. Pre- and postincubation viable colony counts were compared in order to calculate log_{10} killing. On the basis of our previous study (6), serum sensitivity was defined as ≥ 1.0 log_{10} (90%) killing, resistance as < 0.1 log_{10} killing, and intermediate, between these two values; all strains were tested at least in duplicate.

Consumption of hemolytic complement activity. Consumption of total hemolytic complement activity was measured in reaction mixtures of 100% pooled NHS containing 10^9 CFU/ml of serum-sensitive or serum-resistant organisms harvested and then pelleted from 24-h cultures on BAP plates incubated at the temperature optimum for that strain. Hemolytic titrations on reaction mixture supernatants were performed after incubation at 37°C for 30 or 60 min. The standard hemolytic assay using sheep erythrocytes and rabbit serum was performed as described previously (6). Controls included serum alone without bacteria, incubated and handled in parallel with the test samples. The serum-sensitive *E. coli* strain was used as a positive control.

Consumption of C3, C5, and C9. From 10^8 to 10^9 bacterial CFU from 24-h *C. fetus* cultures were incubated at 37°C with NHS for 120 min on a rotater. At designated times, an aliquot of the reaction mixture was withdrawn, immediately plunged into an ice bath, and the bacterial cells pelleted by centrifugation at 12,000 *g* for 2 min, and each supernatant quick-frozen at −70°C. Consumption of hemolytic C3, C5, and C9 from the supernatant was measured by using published procedures (15). The percent consumption in experimental samples was determined relative to control serum samples without bacteria, incubated in parallel with the test samples.

Purification and radiolabeling of complement components. C3 and C9 were purified from fresh human plasma by using modifications of the procedure published by Hammer (15, 16). Both preparations gave single bands on SDS-PAGE and a single line on double diffusion when tested with antisera to whole human serum. The specific hemolytic activities of the purified preparations were as follows: for C3, 117 U/μg (serum 1.11×10^5 U/ml); and for C9, 746 U/μg (serum 4×10^4 U/ml). C3 and C9 were radiolabeled with ^{125}INa by using iodobeads to specific activities of 4.7×10^5 cpm/μg for C3 and 9.3×10^5 cpm/μg for C9. For some experiments, C3 was labeled with NaB $^3H_4$ by reductive methylation to a specific activity of 2.8×10^6 cpm/μg.

Binding of C3 and C9. Binding of C3 and C9 to the bacterial surface was measured as described previously (15). Briefly, mixtures of serum and bacteria were prepared as described above for the serum bactericidal assay, except that the bacterial inoculum was raised to 10^8 to 10^9 CFU/ml, and that either ^{125}I-C3, [^{3}H]C3, or ^{125}I-C9 was added to the mixture. Samples were incubated at 37°C. At designated intervals, 200-μl samples were removed and added to microcentrifuge tubes containing 1 ml of cold HBSS, and the tubes were centrifuged for 5 min at 12,500 *g*. The supernatants were aspirated completely, and the pellets were counted in a gamma counter. For determining binding when [^{3}H]C3 was used, pellets were boiled in 1% SDS for 5 min, scintillation fluid (Opti-fluor, Packard Instrument Co., Downers Grove, IL) added, and counted in a beta counter. Controls for nonspecific binding were bacteria that were incubated in serum previously heated for 30 min at 56°C (HINHS) to block complement activation. Molecules of C3 and C9 bound to the bacterial surface were calculated as described previously (15). ^{125}I-C9 was eluted from the bacterial cells by repeated washing as described (17) except that Medium 199 was used. The molecular form of C3 bound to the test bacteria was determined after release of covalently bound C3 from acceptor molecules by hydroxylamine cleavage, followed by SDS-PAGE and autoradiography as described (18, 19). To determine whether *C. fetus* strains had intrinsic protease activity, [^{3}H]C3 in Medium 199 buffer was incubated for 60 min at 37°C with 10^8 to 10^9 CFU of 82-40 LP or 82-40 HP, and then resolved by SDS-PAGE and autoradiography. The negative control was [^{3}H]C3 incubated in buffer alone, while for the positive control [^{3}H]C3 was incubated with 82-40 HP cells in the presence of 10% NHS.

Preparation of human PMN. 20 ml of peripheral venous blood from one of several healthy donors was collected in a syringe with 6 ml of dextran (6% wt/vol) in 0.9% saline with 300 U of heparin and erythrocytes sedimented at 37°C for 1 h essentially as described (20). The granulocyte-rich fraction was decanted, centrifuged at 436 *g* for 5 min, the pellet was washed once in heparinized (1 U/ml) normal saline and resuspended in HBSS with 1% gelatin (Hank's gel, pH 7.4). The cells (75–80% PMN) were counted in a hemocytometer, the concentration of the suspension was adjusted to 10^7 PMN/ml, and cells were used within 30 min of purification. Viability was > 99% by the criterion of trypan blue exclusion.

PMN killing assay. The test bacteria were harvested from overnight growth (4 h for *S. aureus*) on BAP at 37°C and washed once in sterile distilled water (SDW). A pellet of 10^8 to 10^9 CFU was resuspended in SDW, pelleted at 12,500 *g* in sterile microfuge tubes, and incubated

with 100 μl of the test serum at 37°C for 30 min on a rotator at 15 rpm as described (21). The preopsonized bacteria were then pelleted by centrifugation, resuspended to 450 μl in Hank's gel to yield a final concentration of 10^7 to 10^8 CFU/ml when combined with 100 μl HINHS, and 450 μl of Hank's gel with or without PMN. The tubes were incubated on a rotator at 37°C for 2 h in ambient atmosphere. Immediately and at 1 and 2 h, 100-μl aliquots were withdrawn, serially diluted by tenfold and inoculated on BAP, and plates were incubated for enumeration of viable bacterial colonies.

Phagocytosis. Phagocytic studies were based on the percent association of ^{32}P-labeled *C. fetus* cells with PMN. Bacteria were grown in brucella broth with 500 μCi of ^{32}P (New England Nuclear, Boston MA) for 18 h at 37°C in a microaerobic atmosphere. The cells were washed twice in SDW and the pellet resuspended in Hank's gel to a concentration of 10^8/ml. The phagocytosis assay was done at 37°C for 60 min according to the methods of Gupta et al. (20). The reaction mixture contained equal (0.25 ml) parts of the serum used for opsonization, the bacteria, the PMN in Hank's gel at a final concentration of 10^7/ml and Hank's gel alone. Experiments were done using the following serum sources: NHS, HINHS, no serum, or immune serum to either 82-40 LP or 82-40 HP as described above. As a control, no PMN were used in a parallel assay. The reaction was started by the addition of the PMN suspension and stopped by immersion into ice water. The PMN were separated from the bulk of the noningested bacteria by centrifugation at 436 *g* for 5 min, washed free of adhering bacteria, and the cell-associated radioactivity was determined. Viable bacterial counts of the original inoculum, supernatant, and pellet were determined, by plating serial dilutions of each in duplicate.

Electron microscopy. Electron microscopy was performed on preparations of PMN after the phagocytosis experiments according to the methods of Iwata et al. (22). After being washed twice in HBSS, the pellet was fixed for 2 h in 2% (vol/vol) glutaraldehyde buffered in 0.1 M phosphate (pH 7.2), washed in the same buffer, and postfixed in 1% (wt/vol) osmium tetroxide. The pellet was dehydrated and thin sections and grids were obtained as described (22) and resolved on a Philips 300 electron microscope.

Chemiluminescence. Bacteria were preopsonized as in the PMN killing assays except in 200 μl of serum. PMN were harvested by sedimentation in Hetastarch followed by a Percoll gradient as previously described (23, 24), and used at a final concentration in HBSS of 10^6/ml. The reaction mixture, in polystyrene tubes (Sarstedt No. 55.484, Sarstedt, Princeton, NJ) with 2 × 5-mm microstir bars, consisted of 20 μl HINHS, 20 μl 1 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione [Sigma Chemical Co., St. Louis, MO]), 20 μl PMN, 50 μl HBSS, and 100 μl of the preopsonized bacteria. Assays were performed at 37°C using a Picolite Luminometer (Packard Instrument Co.) with 15-s readings every 4 min for 1 h. Calculations were standardized to counts/PMN cell per min as described (24).

Results

Effect of encapsulation on serum susceptibility of Campylobacter fetus. To investigate the role of encapsulation on serum susceptibility of *C. fetus,* the 100,000-mol wt surface protein containing strains and their spontaneously mutated 100,000-mol wt protein-lacking daughter strains (Fig. 1) were incubated in pooled NHS. All strains were LPS serotype A. Confirming our earlier report (7), the encapsulated *C. fetus* strains 82-40 LP and 23D were resistant to killing by 10% NHS whereas their unencapsulated spontaneous mutant strains, 82-40 HP and 23B, were sensitive (Table I). As previously reported (6, 7), neither encapsulated strains was killed in the presence of 10% homologous immune rabbit serum. The *Campylobacter* strains of other LPS serotypes and lacking the 100,000-mol wt proteins were serum-sensitive (Table I).

Complement utilization by serum-resistant and serum-sensitive Campylobacter strains. To understand the mechanism for serum-resistance by *C. fetus* we initially addressed the question of whether a serum-resistant *C. fetus* strain was capable of consuming hemolytic complement activity. *Campylobacter* strains or a known serum-sensitive *E. coli* strain were incubated with NHS and residual CH_{50} activity assessed (Table II). After 30 min of incubation, two serum-sensitive *C. jejuni* strains, the serum-sensitive *E. coli* strain, and the serum-resistant *C. fetus* strain produced similar consumption of CH_{50} activity and after 60 min, activity was below the lower limit of detection. Thus, the serum-resistant *C. fetus* strain consumed total hemolytic complement to a similar extent as did the serum-sensitive *E. coli* and *C. jejuni* strains.

C3 consumption by Campylobacter cells. To specifically address the question of the role of the capsule in serum-resistance of *C. fetus* and to begin an analysis of the location of the block in complement-mediated lysis, we next incubated the two serum-resistant encapsulated *C. fetus* strains, the serum-sensi-

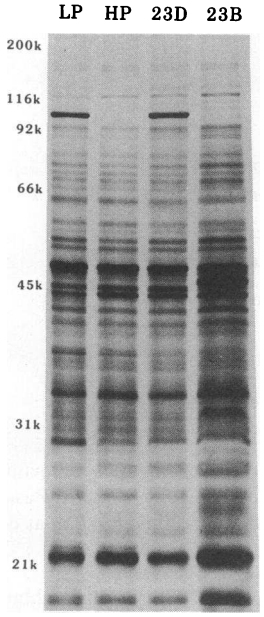

Figure 1. SDS-PAGE (10% acrylamide) of whole cell profiles of *C. fetus* strains 82-40 LP (LP), 82-40 HP (HP), 23D, and 23B. The presence of a major band migrating at 100,000 mol wt can be seen in the profiles of the serum-resistant 82-40 LP and 23D cells but not in the profiles of the two mutant strains. k, mol wt in thousands.

Table II. Complement Utilization by Serum-sensitive and Serum-resistant Strains

Bacterial strain incubated with pooled NHS	Serum susceptibility of strain*	Residual CH_{50} after incubation at 37°C for 30 min	60 min
		unit/ml	
NHS alone	—	45	45
Heat-inactivated serum	—	<10‡	ND§
C. jejuni 81-93	S	32	<10
C. jejuni 79-193	S	31	<10
C. fetus 82-40 LP	R	40	<10
E. coli K12	S	22	<10

* Serum susceptibility based on $\log_{10}$ kill in standardized bactericidal assay with pooled normal serum (6). For specific results, see Table I.
‡ Limit of detection.
§ Not done.

*Table III. Effect of Encapsulation of Campylobacter Cells on Consumption of C3 in Normal Serum**

Bacterial strain	Presence of 100,000-mol wt protein capsule	Log_{10} CFU	Percent C3 consumed 15 min	Percent C3 consumed 120 min
C. jejuni 79-193	−	8.49	10.2 (1.20)	41.0 (4.83)
C. fetus 23D	+	8.44	0 (0)	56.8 (6.73)
C. fetus 23B	−	8.10	11.0 (1.36)	42.2 (5.21)
C. fetus 82-40 LP	+	8.04	35.0 (4.35)	57.5 (7.15)
C. fetus 82-40 HP	−	8.54	38.4 (4.49)	47.3 (5.54)

* Isolates at 1.3–3.5 × 10^8 CFU/ml were incubated in pooled NHS at 37°C. Residual C3 hemolytic activity was assayed at 15 and 120 min as described (15). Percent C3 consumed, expressed relative to the control tubes containing PNHS without bacteria, is the mean for three determinations. Numbers in parentheses indicate percent consumption per log_{10} bacterial CFU.

tive unencapsulated *C. fetus* strains, and a (control) serum-sensitive *C. jejuni* strain with pooled NHS and measured C3 consumption (Table III). By 120 min, incubation with the bacterial cells produced consumption of 41–57% of C3 activity compared with a control serum without bacteria incubated. The serum-resistant strains consumed the same amount of C3 as did the serum-sensitive strains, a reproducible finding in three separate experiments. From these studies we concluded that differences in C3 consumption could not explain the marked differences in serum susceptibility of the strains.

C5 and C9 consumption. To determine whether serum resistance was mediated at a later point in the complement cascade, we next examined consumption of C5 and C9, two complement components associated with the bacteriolytic terminal membrane attack complex. For *C. fetus* strain 23D consumption of both C5 and C9 were markedly impaired when compared with strain 23B (Fig. 2); the extent of the defect in consumption for strain 23D was parallel for C5 and C9. In a second experiment, incubation of *C. jejuni* 79-193 in normal human serum depleted C9 activity by 95% in 30 min and completely within 60 min. In contrast, incubating *C. fetus* 82-40 LP in the same serum resulted in only 30% and 40% depletion at 30 and 60 min, respectively. Substitution of *C. fetus*-immune rabbit serum produced more rapid consumption of C9 by *C. fetus* than did normal serum but did not significantly increase total consumption at 60 minutes.

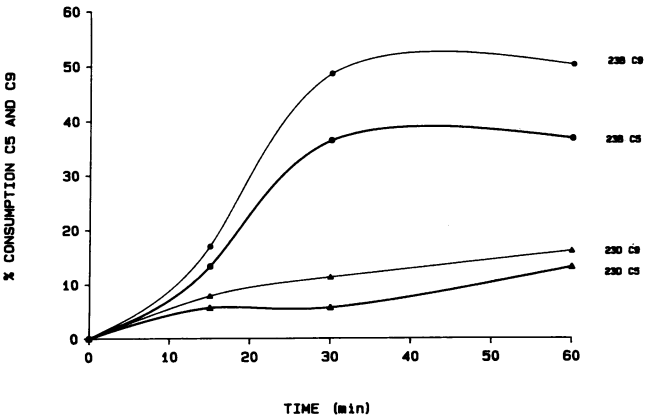

Figure 2. Consumption of C5 and C9 by *C. fetus* strains 23D and 23B. Approximately 10^9 bacterial cells were incubated with pooled NHS at 37°C. At various intervals, aliquots were withdrawn from the mixture, the suspension was centrifuged at 12,000 *g*, and an assay for C5 and C9 functional hemolytic activity was performed on the supernatant. Each point represents the mean of duplicate determinations.

C9 binding. To determine if the differences in terminal component consumption were reflected in differences in C5b-9 binding, we measured deposition of C9 on sensitive and resistant strains. In the presence of normal serum, specific C9 deposition on serum sensitive, unencapsulated strains, was rapid and extensive, peaking by 30–60 min. The amount of C9 bound after 30 minutes was 3- to 10-fold higher than on the serum-resistant, encapsulated strains. Results are shown in Fig. 3 for C9 binding to 79-193 and 82-40 LP, but were qualitatively similar for the other strains. Substitution of immune rabbit serum for normal rabbit serum neither rendered 82-40 LP susceptible to killing nor altered C9 binding. These results indicate that the differences in C9 consumption between the serum-sensitive and serum-resistant strains are reflected in differences in C5b–9 binding to the organisms.

We next examined the nature of the interaction between C5b-9 and the bacterial surface. Cell pellets bearing C5-^{125}I-C9 were serially washed in buffer to determine how readily the bound ^{125}I-C9 was eluted. After three washes over 30 min, a mean of 19.8% of counts were eluted from *C. jejuni* 79-193 whereas 51.7% were eluted from *C. fetus* 82-40 LP despite far less C9 binding to the latter in the original assay (Fig. 3). The major differences occurred during the first 5-min wash in which 17% and 44% of counts were removed from 79-193 cells and 82-40 LP cells, respectively. Subsequent washing removed

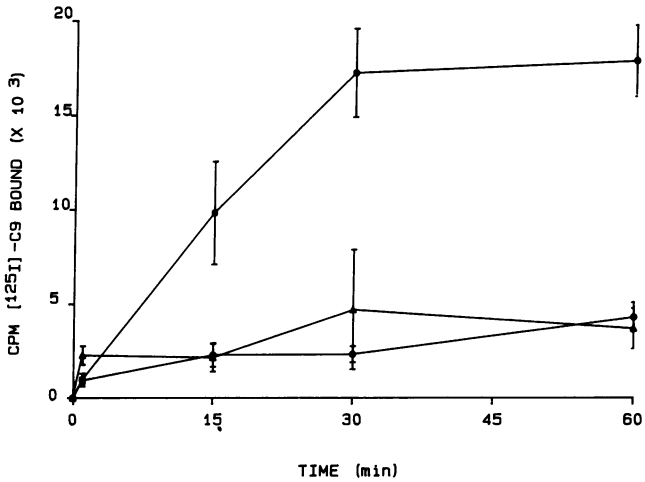

Figure 3. Kinetics of ^{125}I-C9 binding to *C. jejuni* 79-193 and *C. fetus* 82-40 LP. Washed bacterial cells were incubated with pooled NHS or immune rabbit serum to 82-40 LP, and equal amounts of biologically active ^{125}I-C9 were added. At various time intervals, suspensions were centrifuged at 12,000 *g*, supernatants were saved, and ^{125}I counts in the pellet were determined. Specific C9 binding was calculated by subtracting values for incubation in heat-inactivated serum from values in intact serum. Each point represents the mean (± standard error) of two to four replicate assays. Log_{10} bacterial cfu used were 9.3±0.5 for *C. jejuni* 79-193 in NHS (○), 8.8±0.2 for *C. fetus* 82-40 LP in NHS (●), and 9.2±0.4 for *C. fetus* 82-40 in IRS (△).

approximately the same low fraction of counts from both pellets. The results are consistent with the concept that a large portion of C5b-9 bound to the serum-resistant strains is not inserted into hydrophobic domains of the outer membrane, suggesting that there is a qualitative as well as a marked quantitative defect.

C3 binding. Although consumption of C3 by the encapsulated strains was equivalent to consumption by the unencapsulated strains, the limited C5 and C9 consumption and C9 binding on the former group suggested that formation of the C5 convertase on the surface of these cells was defective. To test this possibility, and because C3 is an obligatory component of the C5 convertase, we compared binding of either ^{125}I- or ^{3}H-labeled C3 to the encapsulated and unencapsulated strains. For both encapsulated strains, C3 binding in the presence of NHS was markedly impaired compared with the unencapsulated strains. Results comparing binding to strains 23B and 23D are shown in Fig. 4. Although incubation with immune serum permitted a small increase in C3 binding to encapsulated strain 82-40 LP, it did not correct the substantial defect in comparison to the unencapsulated strain (Fig. 5).

Molecular form of bound C3. The biological activity of bound C3 depends on the molecular configuration of the bound fragment. Although the previous work showed that there was a marked quantitative difference in C3 binding to the encapsulated and unencapsulated strains, we next considered whether there was a qualitative difference as well. To accomplish this, we examined the form of C3 deposited on the encapsulated and unencapsulated strains, in this case, 23D and 23B. To determine which C3 components bound to the cell surface, bacterial cells that had been incubated with ^{125}I-C3 were washed, treated with hydroxylamine to release covalently bound C3, radioactive counts in the supernatants equalized and proteins resolved by SDS-PAGE and autoradiography (Fig. 6). On 23B, the alpha prime chain fragment (115,000 mol wt) indicative of C3b predominated over the alpha chain fragment (67,000 mol wt) indicative of the hemolytically inactive cleavage product, iC3b. In contrast, for strain 23D, more iC3b is present as reflected by a relatively greater proportion of label present in the 67,000-mol wt fraction. In addition, a large

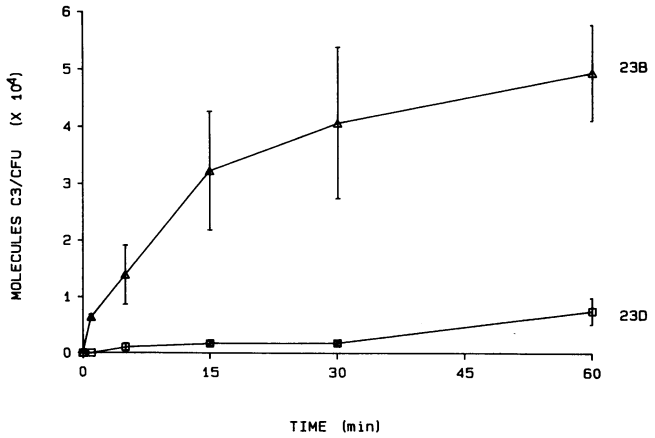

Figure 4. Characteristics of ^{125}I-C3 binding to *C. fetus* 23B and 23D. Bacterial cells were incubated in pooled NHS or heat-inactivated pooled NHS as a control and methods were as specified in legend for Fig. 3.

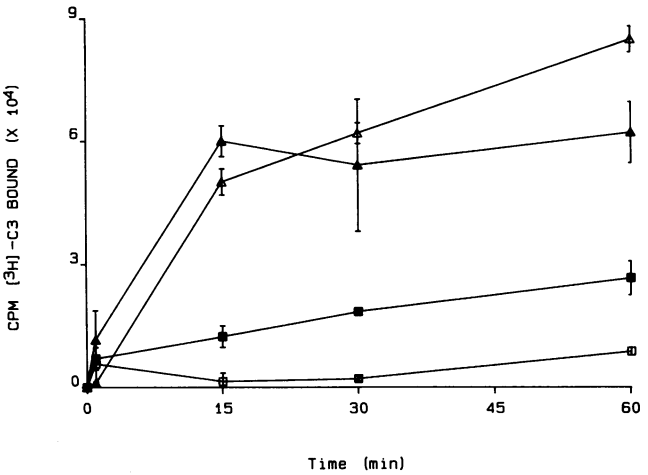

Figure 5. Characteristics of [^{3}H]C3 binding to *C. fetus* 82-40LP (*boxes*) and 82-40HP (*triangles*). Bacterial cells were incubated in normal rabbit serum (*open symbols*), or immune rabbit serum to 82-40LP (*closed symbols*). Methods were as specified in legend to Fig. 3.

amount of C3dg (41,000-mol wt), representing further degradation of the C3, also is present. The proportion of beta chain (75,000 mol wt) bound for the two strains was similar, and similar to the proportion seen in native C3, as expected. The poorly resolved regions below 41,000 mol wt on strains 23B and 23D do not correspond to any known C3 fragments. By laser densitometry of three separate determinations, the ratio (mean±SD) of the hemolytically active C3b (115,000 mol wt) to the hemolytically inactive fragments, iC3b and C3dg (67,000 and 41,000 mol wt) for *C. fetus* 23B was 1.47±0.044 and for 23D was 0.17±0.01 (paired *t* test, $P < 0.001$), a more than eightfold difference. These results indicate that the small amount of C3b initially bound to strain 23D is present pre-

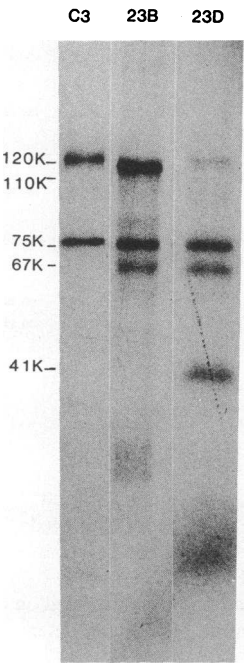

Figure 6. C3 fragments bound to *C. fetus* strains 23D and 23B. After ^{125}I-C3 was bound in the presence of pooled NHS, the bacterial cell pellets were washed, boiled in SDS, and then treated with hydroxylamine to cleave the covalent linkage between C3 and bacterial constituents. Radioactive counts were equalized and proteins were resolved by SDS-PAGE followed by autoradiography. Intact C3 (120,000-mol wt alpha chain, 75,000-mol wt beta chain) also was run as a control. As with other serum-sensitive strains, new 115,000- and 67,000-mol wt proteins representing respectively C3b and iC3b were found on the surface of strain 23B with the hemolytically active C3b residues predominating. Although the amount of C3 bound to 23D was significantly less than to 23B (Fig. 4), the majority of C3 bound to 23D was the hemolytically inactive iC3b. A major band migrating about 41,000 mol wt on 23D represents further cleavage of iC3b to C3dg.

dominantly at sites leading to further cleavage to hemolytically inactive products (iC3b, C3dg) which cannot participate in C5 convertase formation. These experiments indicate a qualitative as well as quantitative difference in C3 binding to the encapsulated strain.

One explanation for the dissociation between C3 consumption and binding observed would be direct proteolysis of C3 by the capsular protein. To address this question, radiolabelled C3 was incubated with *C. fetus* cells and appropriate controls. Incubation of [^{3}H]C3 with high concentrations of encapsulated or unencapsulated cells or with buffer did not result in any proteolysis of C3 in the absence of serum, as determined by SDS-PAGE and autoradiography. Typical proteolysis occurred in the presence of *C. fetus* cells and 10% NHS, as expected. Thus, the capsular protein is not directly proteolytic to C3.

Influence of opsonin source on bactericidal activity of PMN toward C. fetus strains. Since the amount and form of bound C3 influences its function as an opsonin, the following experiments were designed to provide answers to two basic questions: first, are PMN bactericidal to *C. fetus* strains, and second, how does the presence of the capsular protein affect this phenomenon? Initial studies utilized *Staphylococcus aureus* 502A which is known to be killed in the presence of PMN (21). At a 6:1 ratio of bacteria to PMN in the presence of HINHS, we found > 1-$\log_{10}$ killing of this organism by 60 min, whereas no killing was observed in the absence of PMN, as expected, (Fig. 7 *A*). Under identical assay conditions, we found that for the unencapsulated strain 82-40 HP opsonized with HINHS there was greater than 2-$\log_{10}$ killing in the presence of PMN but not in their absence (Fig. 7 *B*), indicating that these organisms are readily killed by PMN. Since HINHS was used for these assays, these results show that complement activation is not necessary for opsonization of the unencapsulated strain. In contrast, the encapsulated strain, 82-40 LP, was not killed by PMN in the presence of HINHS or NHS (Fig. 7, *C* and *D*). Preopsonization of this strain by immune serum permitted PMN killing in the presence of both intact (Fig. 7 *E*) and heat-inactivated serum (Fig. 7 *F*), suggesting that specific antibody alone was sufficient for opsonization. Nearly identical killing was observed when 82-40 LP was preopsonized with heat-inactivated immune serum to *C. fetus* 82-40 HP or *C. jejuni* 79-193 (data not shown). Since these organisms do not possess the S-proteins but share flagellar, outer membrane

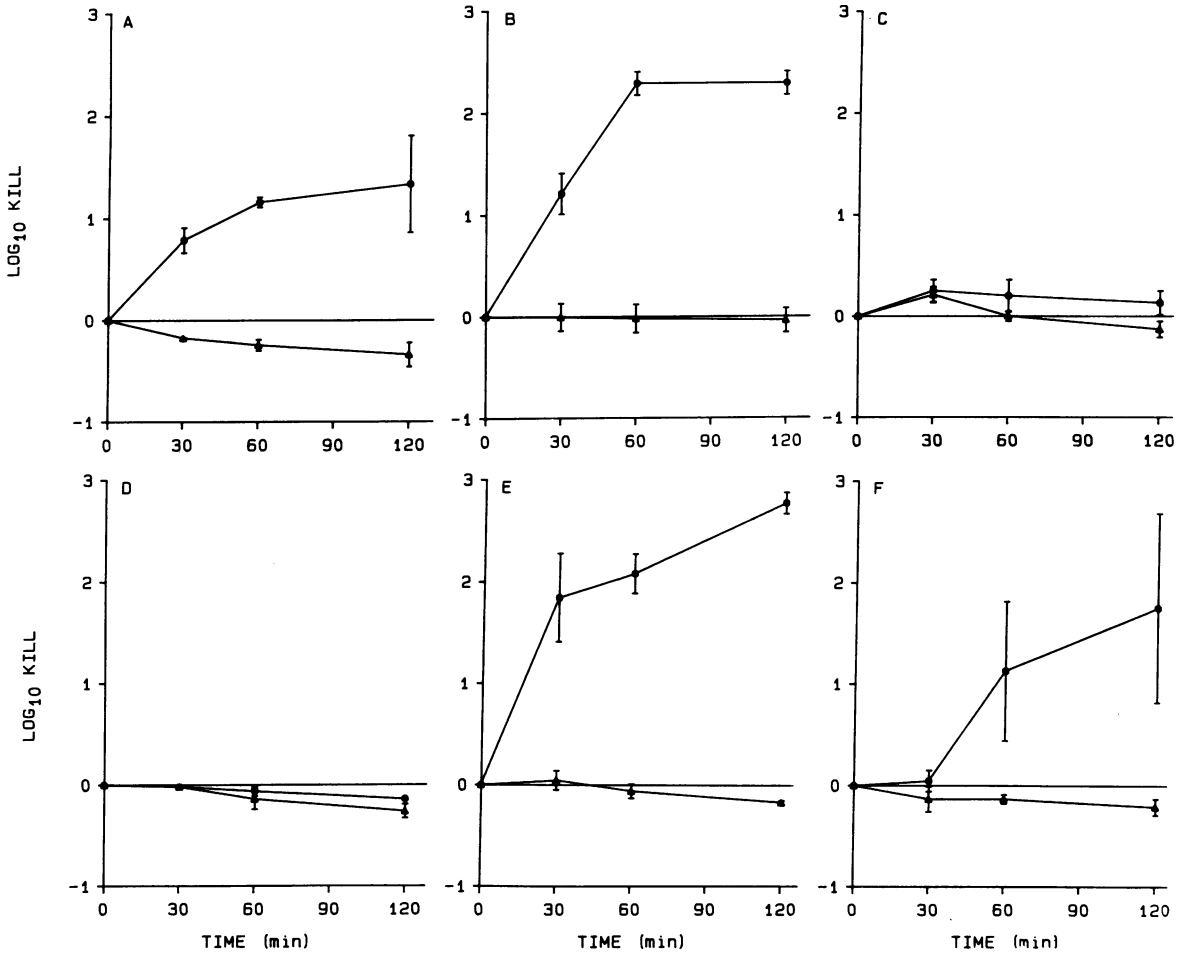

Figure 7. Killing of *C. fetus* strains 82-40 LP and 82-40 HP (and *S. aureus* 502A) by PMN. In each experiment bacterial cells were preopsonized and then incubated with (●) or without (▲) PMN for periods ranging from 0 to 120 min. (*A*) *S. aureus* 502A preopsonized with heat-inactivated NHS. (*B*) *C. fetus* 82-40 HP preopsonized with heat-inactivated NHS. (*C*) *C. fetus* 82-40 LP preopsonized with heat-inactivated NHS. (*D*) *C. fetus* 82-40 LP preopsonized with NHS. (*E*) *C. fetus* 82-40 LP preopsonized with 82-40 LP immune rabbit serum. (*F*) *C. fetus* 82-40 LP preopsonized with heat-inactivated immune rabbit serum.

protein (7) and lipopolysaccharide (11) antigens (82-40 HP) or flagellar antigens (25) only (79-193), it is clear that the epitopes of the antibodies in the immune serum are not critical. Thus, these results indicate that unencapsulated *C. fetus* strains can be killed by PMN in the presence of normal serum, but that the encapsulated organisms resist killing, a defect that can be overcome in the presence of immune serum.

Influence of opsonin source on stimulation of PMN chemiluminescence by C. fetus strains. Because the lack of killing of the encapsulated strain by PMN may have represented either a failure of PMN to phagocytose these organisms or adequate phagocytosis but resistance to intracellular killing, we next examined the ability of *C. fetus* strains to stimulate a phagocytic response. The interaction of bacteria with the cell membrane of PMN elicits a sequence of intracellular events resulted in a marked increase in cellular oxidative metabolism and emission of chemiluminescence (26); further, the extent of the chemiluminescence response has been used to measure bacterial phagocytosis by PMN (27).

Initial studies showed that in the presence of HINHS a ratio of 500 *C. fetus* 82-40 HP CFU per PMN provided maximal chemiluminescence response (Fig. 8 *A*), and that results were nearly identical whether the bacteria were preopsonized or opsonized during incubation with the PMN (data not shown). However, there was minimal stimulation of the chemiluminescence response by *C. fetus* 82-40 LP cells in the presence of HINHS, at the same 500:1 ratio (Fig. 8 *B*). In a subsequent assay, stimulation of chemiluminescence in the presence of HINHS again was negligible whereas a significantly greater response was shown in the presence of immune serum (Fig. 9). Preopsonization with 1.25–100% immune serum produced maximal chemiluminescence response, but absorption of the immune serum six times with the homologous encapsulated strain ablated virtually all of the increment in chemiluminescence response above the baseline produced in the presence of HINHS (data not shown), suggesting that specific antibody mediated the enhanced chemiluminescence response of immune serum. These experiments indicate that in the absence of specific antibody encapsulated *C. fetus* cells are not taken up by PMN or that they have bypassed chemiluminescence-producing mechanisms.

Uptake of C. fetus strains by PMN. To further clarify the reason for the poor chemiluminescence response, we then examined the association of radiolabeled bacteria with human PMN. As expected, at a ratio of bacteria:PMN of 10:1 using ^{32}P-labeled *Staphylococcus aureus* 502A, opsonization with NHS permitted significantly greater cell-association than for nonopsonized bacteria. Using this same assay for ^{32}P-labeled *C. fetus* cells opsonized with HINHS, in the absence of PMN, for both strains essentially no counts were present in the pellet produced by low-speed centrifugation, as expected (Fig. 10, lane *1*). When bacteria were incubated with PMN alone without opsonization, there were low counts in the resulting PMN pellet from the encapsulated strain and from the unencapsulated strain (lane *2*). However, after opsonization with HINHS, there was more than a 19-fold differential in the radioactive counts in the PMN pellets with encapsulated and unencapsulated strains (lane *3*). Opsonization in NHS caused a small rise in the association of the encapsulated strain with PMN from the HINHS values, but for the unencapsulated strain the association diminished (lane *4*). This was due to the fact that in the presence of NHS, there was 100% killing of the

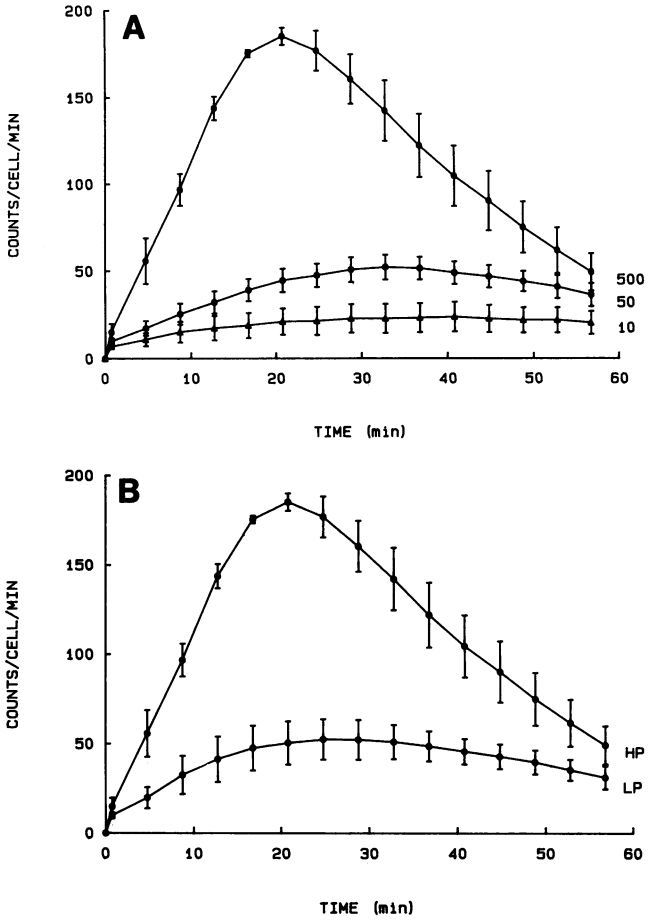

Figure 8. Chemiluminescent responses of human PMN to *C. fetus* 82-40 HP and 82-40 LP. (*A*) The reaction mixtures contained PMN (10^6 cells per ml), *C. fetus* 82-40 HP (ratio ranging from 10 to 500 bacteria per PMN), 10% pooled NHS, and luminol (8.3×10^{-7} M). Each point represents the mean of chemiluminescent responses ± standard deviation from three separate determinations. Preopsonization of cells did not affect results (data not shown). (*B*) Chemiluminescent responses of human PMN to *C. fetus* 82-40 LP and 82-40 HP. Experiments using 500:1 bacteria to PMN ratios are shown. Strain 82-40 LP produced little stimulation at every ratio tested.

unencapsulated (serum-sensitive) strain with disintegration of cells and solubilization of radiolabel. Opsonization of organisms with their homologous immune serum resulted in high association of radiolabel with the PMN pellet (lane *5*); for the encapsulated strain this was markedly greater than occured with normal serum. Thus, failure of the encapsulated strains to elicit chemiluminescene in the absence of immune serum is caused by a lack of association with the phagocytic cells.

To determine the nature of this association, we performed electron microscopy on mixtures of the encapsulated and unencapsulated bacteria opsonized with HINHS. Within 5 min, virtually all of the cells of the unencapsulated strain were seen within phagocytic vacuoles inside the PMN (Fig. 11 *A*). In contrast, essentially all of the encapsulated organisms were extracellular (Fig. 11 *B*); only rare intracellular organisms were seen. As indicated by the chemiluminescent, radiolabel uptake and electron microscopic studies, the mechanism by which the encapsulated strains resist PMN killing appears to be lack of phagocytosis.

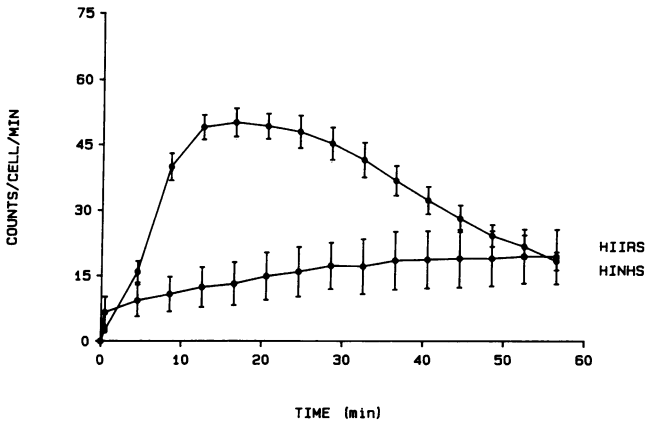

Figure 9. Chemiluminescent responses of human PMN to *C. fetus* 82-40 LP in the presence of HINHS or heat-inactivated immune (82-40 LP) rabbit serum (HIIRS). Ratio of bacteria to PMN is 500:1 and methods are as described in legend to Fig. 7.

Discussion

The experiments have explored the mechanisms of serum and phagocytosis resistance in *C. fetus*. We examined complement component consumption and uptake on the bacterial surface, and phagocytosis and killing of S-protein encapsulated and unencapsulated strains of *C. fetus*. The encapsulated strains were serum-resistant, biochemically typical (28) and isolated from systemic sites in infected hosts, and are representative of *C. fetus* isolates from humans (7). The serum-sensitive *C. fetus* strains were spontaneous laboratory mutants whose major structural difference with the parental strains was the absence of the capsular protein (7). Because these mutations occurred after multiple in vitro passages, in other studies we have used pronase to selectively remove the S-protein capsule from intact cells without effect on any other band or on cell viability (29, 30). In those experiments, the protease-treated cells became susceptible to killing and opsonization by normal serum, confirming the critical role of the capsule in resistance to these host defense mechanisms. This analysis confirmed that the use of the mutant strains allowed us to specifically examine the role of the capsular S-protein in interactions with complement and phagocytic cells.

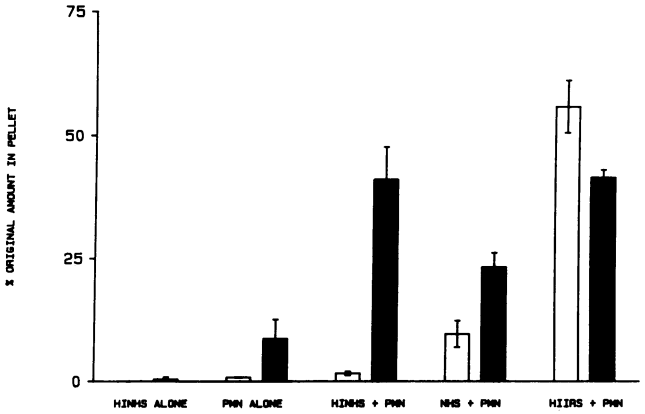

Figure 10. Association of *C. fetus* 82-40 LP (*white bars*) and 82-40 HP (*black bars*) with human PMN. ^{32}P-labeled bacteria were incubated with HINHS alone, or at a 10:1 ratio with PMN alone, with PMN + HINHS, with PMN + NHS, or with PMN + heat-inactivated immune rabbit serum (HIIRS) to 82-40 LP or to 82-40 HP. After 60 min a pellet was obtained by low speed centrifugation, washed once, the number of counts in the (PMN) pellet determined, and compared with number of counts in the original bacterial pellet. Each bar represents the mean (± standard error) for triplicate determinations.

Serum resistance of the encapsulated strains does not reflect a failure of these organisms to initiate complement activation. Depletion of total hemolytic activity and consumption of C3 was equivalent for encapsulated and unencapsulated strains. However, minimal consumption of C5 and C9 and C9 binding to the encapsulated strains all suggested that the cascade was interrupted at the step of the C5 convertase. This idea was supported by direct experiments demonstrating that little C3 binds to the encapsulated strains, thereby precluding efficient C5 convertase formation. Furthermore, most of the C3 bound is in the form of the hemolytically inactive iC3b fragment which cannot participate in the cleavage of C5 nor lead to formation of the C5b-9 membrane attack complex. Thus, the defect is qualitative as well as quantitative. The predominence of iC3b indicates that binding occurred in a "nonprotected" site, permitting rapid cleavage by serum control proteins, H and I (31). Lack of C3b binding (and later C9 binding) even in the presence of immune serum is consistent with our earlier observations of serum resistance even in the presence of specific antibodies (6, 7), which differentiates *C. fetus* from most serum-resistant *Enterobacteriaceae* (32). That substituting immune for normal serum made little difference either in serum-susceptibility (6) or in C3 or C9 binding suggests that antibody-directed complement binding to cell surface sites is not important for the encapsulated serum-resistant *C. fetus* strains.

Our data are consistent with the hypothesis that failure of C3b binding to the encapsulated strain is the central mechanism for serum resistance, although the mechanism for C3 consumption without binding to these organisms is presently unknown. Binding of C3 but with rapid release is one possible mechanism; however, binding determinations done after only 5 min of incubation do not support this hypothesis. The capsular protein may have protease activity for C3 or C3 fragments but we could find no evidence for this. Alternatively, the capsule may preclude covalent attachment of C3 to the bacterial surface which would not permit amplification by the alternative pathway (33). In any event, the mechanism of serum-resistance of encapsulated *C. fetus* appears to be novel and differs from those described for smooth *Enterobacteriaceae* (15), or serum-resistant gonococci (34, 35). For those organisms, C3 binding and C5b-9 generation are efficient, but for a variety of reasons (18, 34) the terminal membrane attack complex fails to insert into vital cell membrane structures. The exact mechanisms involved in the novel form of complement-resistance by encapsulated *C. fetus* cells deserve further examination.

Encapsulated *C. fetus* strains resist PMN killing except in the presence of immune serum, confirming and extending the observations of McCoy et al. (8). Antibody to capsule per se is not necessary, as killing occurs in the presence of antibody to other *Campylobacter* strains which lack the S-proteins but which share other surface determinants (7, 11, 25). We used four different assays of phagocytic activity—bactericidal, chemiluminescence, association with radiolabeled bacteria, and

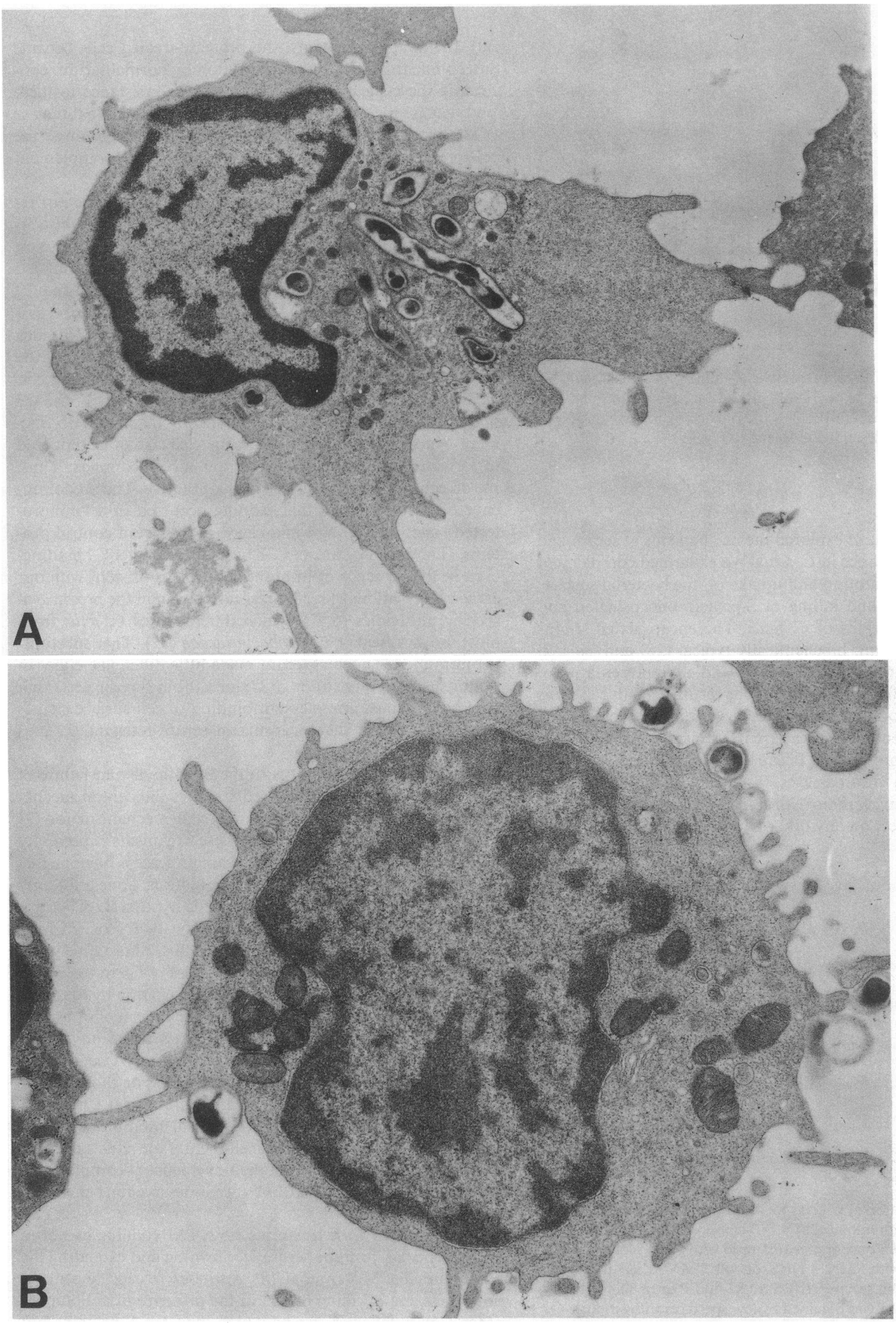

Figure 11. Electron photomicrographs of PMN incubated with *C. fetus* 82-40 HP (*A*) and 82-40 LP (*B*) plus HINHS at 37°C for 5 min. PMN have ingested essentially all 82-40 HP cells which are present in phagocytic vacuoles whereas virtually all of the 82-40 LP cells that are associated with the PMN are extracellular.

electron microscopy. Results of all experiments were consistent with the hypothesis that resistance to killing is due to the ability of encapsulated strains to resist phagocytosis, but if properly opsonized, these organisms are readily killed. The parallel anti-opsonic properties of capsules of other serum-resistant bacteria including pneumococci (36), and *Vibrio vulnificus* (37) have been well described. Not surprisingly, unencapsulated *C. fetus* cells which share core LPS antigens with other gram-negative organisms (38), are readily phagocytosed and killed by PMN.

The failure of encapsulated *C. fetus* strains to be killed by PMN in the presence of NHS also is consistant with defective C3 binding. iC3b is a more important opsonin of encapsulated pneumococci than is C3b (39). Although iC3b is the predominant form deposited on encapsulated *C. fetus* strains, the very small amounts of C3 derivatives deposited in toto are apparently unable to stimulate phagocytosis via either the CR1 or CR3 receptors. That heat-inactivated immune serum is fully opsonic whereas NHS is not, suggests that phagocytosis is occurring via the Fc but not the C3 receptors of the PMN (40). Thus, encapsulation potentially provides important advantages for *C. fetus* cells in mammalian hosts. The host may destroy unencapsulated *C. fetus* organisms by either serum or phagocytic cells, but must wait for development of specific antibodies to permit destruction of these encapsulated organisms by phagocytic cells. This model helps explain why nearly all *C. fetus* infections of humans are caused by encapsulated strains (7). Conservation of amino acid composition, antigenicity, and amino-terminal sequence for the S-layer proteins derived from a variety of strains (41) further indicates the biological relevance of these molecules.

Acknowledgments

We thank Dr. Peter Kohler for assessing total hemolytic complement activity, Ann Tartanian for performing individual complement titrations, Jan Hopkins for gel electrophoresis, Elaine Berger for assisting with the chemiluminescence assays, and Rolf Dahl for providing the electron photomicrographs.

This work was supported in part by the U. S. Army Medical Research and Development Command and by the Medical Research Service of the Veterans Administration. Dr. Blaser is a Clinical Investigator of the Veterans Administration.

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