Pathogenesis of *Campylobacter fetus* Infections

Role of Surface Array Proteins in Virulence in a Mouse Model

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Abstract

We developed a mouse model to compare the virulence of *Campylobacter fetus* strains with (S-plus) and without (S-minus) surface array protein (S-protein) capsules. In adult HA/ICR mice pretreated with ferric chloride, the LD_{50} for S-plus strain 84-32 was 43.3 times lower than its spontaneous S-minus mutant 84-54. Seven strains of inbred mice were no more susceptible than the outbred strain. In contrast to the findings with *Salmonella typhimurium* by others, 3×10^7 CFU of strain 84-32 caused 90% mortality in C3H/HeN (LPS^n) mice and 40% mortality in C3H/HeJ (LPS^d) mice. High-grade bacteremia in HA/ICR mice occurred after oral challenge with S-plus *C. fetus* strains and continued for at least 2 d, but was not present in any mice challenged with S-minus strains. Bacteremia at 30 min after challenge was 51.6-fold lower in mice pretreated with 10 μl of rabbit antiserum to purified S-protein than after pretreatment with normal rabbit serum. Challenge of mice with a mixture of S-minus strain 84-54 and free S-proteins at a concentration 31.1-fold higher than found in wild-type strain 84-32 caused 30% mortality, compared with 0% with strain 84-54 or S-protein alone. These findings in a mouse model point toward the central role of the S-protein in the pathogenesis of *C. fetus* infection. The S-protein is not toxic per se, but enhances virulence when present on the bacterial cell surface as a capsule. (*J. Clin. Invest.* 1990. 85:1036–1043.) surface array proteins • *C. fetus* animal model • *C. fetus* infection • *C. fetus* bacteremia • *C. fetus* virulence factor

Introduction

Campylobacter fetus subspecies *fetus* is recognized in humans as an opportunistic pathogen causing systemic infections in immunocompromised hosts (1–4). Recent reports indicate that *C. fetus* also causes diarrhea and suggest that the intestinal tract may be its portal of entry into the bloodstream (5). Although the mechanisms by which *C. fetus* causes systemic infection have not been defined, several in vitro studies indicate that a surface array protein (microcapsule) in wild-type *C. fetus* strains plays a critical role in the organism's ability to resist both the complement-mediated bactericidal activity in normal human serum and phagocytosis by neutrophils (6–8). This serum- and phagocytosis-resistance is the probable explanation for the high frequency of extraintestinal infection by *C. fetus*.

C. fetus cells contain several surface array proteins (S-proteins)[1] with molecular weights ranging from 100,000 to 149,000 (9), which form a hexagonal subunit capsular structure on the outer surface of the cell (10). For a given strain, a single protein usually is predominant (9). After repeated in vitro passage, some isolates lose these S-proteins and become unencapsulated, without change in other proteins (6, 7). Previous studies of these unencapsulated mutants showed that loss of these high molecular weight S-proteins was associated with loss of resistance to phagocytosis and normal serum killing (6, 7, 11). To date, correlation of these in vitro results with virulence in an animal model has not been demonstrated.

The aim of this study was to establish an animal model for *C. fetus* infections, and then to determine the importance of the S-proteins in *C. fetus* virulence. Using a mouse model, we have now been able to: (*a*) demonstrate that the LD_{50} of a *C. fetus* wild-type strain is significantly lower than that of its unencapsulated mutant; (*b*) correlate the presence of S-proteins on *C. fetus* strains with bacteremia after oral challenge; and (*c*) determine the relative toxicity of the S-protein.

Methods

Bacterial strains. 14 *C. fetus* strains from the culture collection of the Denver Veterans Administration Medical Center Campylobacter Laboratory were used in this study. These strains included 12 original isolates from infected humans or animals and two laboratory mutants. The LPS serotyping and the susceptibility of all these strains to the bactericidal activity present in normal human serum had been previously determined (7, 8, 12). Nine strains contained S-protein, five (84-32, 82-40LP, 84-86, 80-109, and 84-112) were serum resistant ($\log_{10}$ killing < 0.1), and four (84-91, 84-94, 84-107, and 84-108) were serum sensitive ($\log_{10}$ killing > 1). Three strains (81-170, 83-88, and 84-90) were wild-type strains lacking S-proteins and were serum sensitive. Strains 84-54 and 82-40HP were spontaneous mutants of 84-32 and 82-40LP that lacked S-proteins and were serum sensitive. All strains were maintained frozen at −70°C in brucella broth (BBL Microbiology Systems, Cockeysville, MD) containing 15% glycerol. For each study, bacteria were grown on trypticase soy agar with 5% sheep blood (PASCO, Wheat Ridge, CO) in a microaerobic atmosphere (5% oxygen, 10% carbon dioxide, and 85% nitrogen) at 37°C for 48 h for the first two passages, and for 24 h for the third passage.

Animals. The experiments were carried out on female HA/ICR mice (Hsd: [ICR] Br; Harlan Sprague Dawley, Inc., Indianapolis, IN), 6–10 wk old, weighing 20–30 g, and on inbred mice, NZB, C57BL/6J (Jackson Laboratory, Bar Harbor, ME), CBA/N, BAlB/c, B6D2F1, C3H/HeN, and C3H/HeJ (Simonsen Laboratory Inc., Gilroy, CA), 10–30 wk old, weighing 20–30 g. Animals were maintained and all studies were performed according to the National Institutes of Health guidelines for small animals.

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Received for publication 22 February 1989 and in revised form 25 October 1989.

J. Clin. Invest.

0021-9738/90/04/1036/08 $2.00
Volume 85, April 1990, 1036–1043

1. *Abbreviations used in this paper:* IP, intraperitoneal; S-protein, surface array protein; TSBB, Tris/saline blotting buffer.

Analytical procedures. Protein concentrations were measured by using the Markwell et al. modification of the Lowry method (13). SDS-PAGE was performed in a modified Laemmli gel system as described by Ames (14). Proteins were resolved using the modified silver stain of Oakley et al. (15). Molecular weight standards (Bio-Rad Laboratories, Richmond, CA) were as follows: myosin (200,000), B-galactosidase (116, 250), rabbit muscle phosphorylase B (92,500), BSA (66,200), and hen egg white ovalbumin (45,000). The methods of Towbin et al. (16) formed the basis for the Western blot procedure we used. In brief, SDS-PAGE was performed with a gel thickness of 0.75 mm in a Mini PROTEAN II dual slab cell (Bio-Rad Laboratories) at 250 mA for ~ 40 min. The proteins were then transferred from the slab gel to nitrocellulose paper by electroblotting for 2 h at 300 mA. The nitrocellulose paper was then blocked twice (30 min each) in Tris/saline blotting buffer (TSBB) (10 mM Tris base, pH 8.0, 0.5 M NaCl, 0.5% Tween 20, 0.02% NaN_3). The nitrocellulose paper was incubated with a 1:1,000 dilution of serum in TSBB for 40 min. After three washes in TSBB, the nitrocellulose paper was incubated with a 1:5,000 dilution of horseradish peroxidase–conjugated anti–rabbit IgG (Amersham Corp., Arlington Heights, IL). After washing, the nitrocellulose paper was developed in diaminobenzidine solution.

Parenteral challenge. 24-h cultures of *C. fetus* strains 84-32 (S-protein–positive) and 84-54 (S-protein–negative spontaneous mutant) on blood agar plates were harvested in 5 ml of sterile normal saline. Bacterial cells were pelleted at 3,500 *g* for 15 min and resuspended in 0.05 M phosphate buffer with 0.9% sodium chloride, pH 7.4 (PBS). HA/ICR mice were pretreated with 1 or 5 mg of iron as iron dextran or ferric chloride, respectively, or 0.5 ml of normal saline 2 h before intraperitoneal (IP) challenge with fivefold dilutions of bacterial suspensions originally containing 10^{10} CFU in 0.5 ml normal saline. Mice were observed for death daily for 7 d. The LD_{50} for each strain was calculated by the method of Reed and Muench (17). A group of 10 HA/ICR mice was pretreated with 5 mg of iron as iron dextran and 2 h later was intravenously challenged in the tail vein with 10^8 CFU *C. fetus* strain 84-32. For comparison, another group of 10 mice was challenged intraperitoneally with the same dose of organisms. The mortality of the mice was observed for a 7-d period.

Oral challenge. 20-h cultures of *C. fetus* strains on blood agar were harvested, washed in normal saline, and resuspended in PBS as described above. Each mouse was given 1 mg of iron as ferric chloride by the IP route and 2 h later was challenged orally with 0.1 ml of bacterial suspension using a 1-in., ball-tipped, 24-gauge animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY) as previously described (18). Blood samples were collected before challenge and at 0.5, 4, 24, 48, and 120 h after challenge. Each mouse was anesthetized in a jar with a pad of gauze containing metofane (Pitman-Moore Inc., Washington Crossing, NJ) and 200 μl of blood was obtained from the retro-orbital plexus. For each sample, serial dilutions of blood in normal saline were inoculated onto tryptic soy agar plates with 5% sheep erythrocytes (PASCO) and incubated microaerobically for 72 h at 37°C. Colonies with typical morphology for *C. fetus* were counted to determine the bacterial colony-forming units/milliliter of blood. Five colonies were randomly picked from each positive plate and smeared and stained with carbol fuchsin to determine whether the bacteremia was due to a *Campylobacter* species.

Passive protection of mice from bacteremia caused by oral challenge with C. fetus 84-32. A 24-h culture of *C. fetus* S-plus strain 84-32 was harvested with 0.1 M PBS, pH 7.4. 4 h before oral challenge, female 10-wk-old HA/ICR mice were pretreated IP with 10 μl of rabbit antiserum to the 100,000-mol wt S-protein from strain 82-40LP (9), or normal rabbit serum. Both sera had been heated at 56°C for 30 min to inactivate complement. 2 h before challenge mice were pretreated IP with 1 mg iron as ferric chloride. Oral challenge and subsequent blood cultures were performed as described above.

Toxicity of S-protein. S-protein was extracted with water from strain 84-32 as previously described (9). For quantitation of water-extracted proteins from cells of this S-plus strain, bacteria were harvested from 24-h cultures from 10 blood agar plates, and protein concentrations of combined second to fifth water washes (9) and bacterial colony-forming units/milliliter were determined. In total, the amount of protein extracted in this way corresponded to 114 μg/10^{10} CFU. For bacterial challenges, 24-h cultures of strains 84-32 and 84-54 were harvested, washed once with normal saline by centrifugation at 3,500 *g* for 15 min, and resuspended in 5 mM of calcium chloride in 10 mM Hepes buffer, pH 7.2. Subsequently, we added 700 or 3,575 μg of the water-extracted S-proteins from strain 84-32 or equal amounts of BSA as controls to 10^{10} CFU of 84-54 cells. Thus, the S-proteins were present in 6.1- or 31.1-fold greater concentrations with 84-54 cells than with the 84-32 cells from which they were harvested. The HA/ICR mice were pretreated IP with 1 mg iron as ferric chloride and 2 h later challenged IP with strain 84-32 or with the mixture of 84-54 and free S-proteins. Mice were observed for mortality over a 7-d period.

Results

Identification of S-proteins in C. fetus strains. Our initial animal studies were performed with the paired strains 84-32 (23D) and 84-54 (23B), the latter being the spontaneous mutant of the former strain (7, 10). Before use in animals, we confirmed by SDS-PAGE and Western blotting that strain 84-32 possessed the S-protein (S-plus), whereas strain 84-54 (S-minus) did not (Figs. 1 and 2). Also shown in the figure are the SDS-PAGE profiles of the other pair of strains 82-40LP (S-plus) and 82-40HP (S-minus), as well as strains 84-86, 80-109, 84-112, 84-91, 84-94, 84-107, and 84-108 (all S-plus), and 81-170, 83-88, and 84-90 (all S-minus) used in later studies. Western blotting with rabbit antiserum against the 100,000-mol wt S-protein of strain 82-40LP (Fig. 2) confirmed both that the S-proteins were present in these strains and that all were antigenically related, except for the 127,000-mol wt S-protein on strain 84-94. No such proteins or fragments were recognized in the S-minus strains.

Effect of pretreatment on susceptibility to peritoneal challenge. Because *C. fetus* is primarily associated with systemic infection in humans, we first challenged mice with organisms delivered by the parenteral route. The wild-type S-plus strain used in these studies was 84-32. After IP challenge of adult HA/ICR mice with up to 1×10^{10} live bacteria, no mortality was observed (data not shown). Because iron pretreatment enhances virulence of other bacterial pathogens (19–21), we

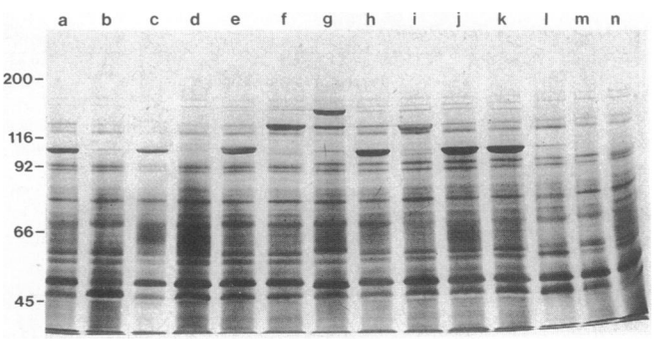

Figure 1. Whole cell proteins of *C. fetus* strains used in this study. SDS-PAGE with 7% acrylamide. Lane *a*, 84-32; lane *b*, 84-54; lane *c*, 82-40LP; lane *d*, 82-40HP; lane *e*, 84-86; lane *f*, 80-109; lane *g*, 84-112; lane *h*, 84-91; lane *i*, 84-94; lane *j*, 84-107; lane *k*, 84-108; lane *l*, 81-170; lane *m*, 83-88; lane *n*, 84-90. Molecular weight markers (in kilodaltons) are shown at left. High molecular weight (100,000, 127,000, and 149,000) S-proteins are present for the strains represented in lanes *a*, *c*, *e*, and *f–k*.

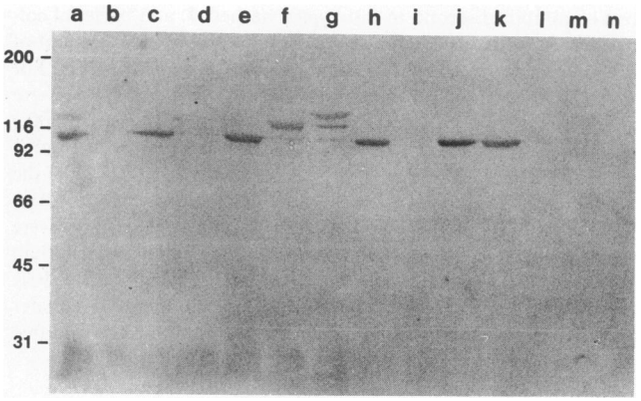

Figure 2. Identification of S-proteins in the whole cell preparation of *C. fetus* strains by immunoblot. Lane *a*, 84-32; lane *b*, 84-54; lane *c*, 82-40LP; lane *d*, 82-40HP; lane *e*, 84-86; lane *f*, 80-109; lane *g*, 84-112; lane *h*, 84-91; lane *i*, 84-94; lane *j*, 84-107; lane *k*, 84-108; lane *l*, 81-170; lane *m*, 83-88; lane *n*, 84-90. First antibody is rabbit anti–100,000-mol wt S-protein of strain 82-40LP. High molecular weight S-proteins were identified by crossreaction with 100,000-mol wt S-protein of strain 82-40LP in the western blot in strain 84-32 (100,000 and 127,000 mol wt) (lane *a*), 82-40LP (100,000 mol wt) (lane *c*), 84-86 (100,000 mol wt) (lane *e*), 80-109 (127,000 mol wt) (lane *f*), 84-112 (127,000 and 149,000 mol wt) (lane *g*), 84-91 (100,000 mol wt) (lane *h*), 84-107 (100,000 mol wt) (lane *j*), and 84-108 (100,000 mol wt) (lane *k*). A 127,000-mol wt S-protein present in strain 84-94 as shown by SDS-PAGE (Fig. 1, lane *i*) was not recognized by antibody to the 100,000-mol wt S-protein of 82-40LP. S-proteins were not found in mutant strains 84-54 and 82-40HP nor in wild-type strains 81-170, 83-88, and 84-90.

next sought to determine whether a similar phenomenon existed for *C. fetus* infection. We found that after pretreatment with either ferric chloride or iron complexed with dextran, intraperitoneally administered strain 84-32 now caused lethality at doses as low as 1×10^7 CFU (Table I). No mortality was seen in mice treated with iron compounds without bacterial challenge. The enhancement due to ferric chloride treatment was greater than that due to iron dextran. IP challenge was associated with greater lethality than was intravenous challenge (Table II). Therefore, based on these studies, we used iron pretreatment and IP inoculation for all subsequent parenteral challenges.

Table I. Effect of Pretreatment with Iron on Susceptibility of Mice to C. fetus Strain 84-32

	Percent mortality after pretreatment*				
	Iron dextran		Ferric chloride		Normal saline
Bacterial dose/mouse	5 mg	1 mg	1 mg	0.2 mg	0.5 ml
1.6×10^9	100	40	100	80	0
1.6×10^8	60	0	100	60	0
1.6×10^7	0	0	60	0	ND
0	0	ND	0	ND	ND

* Iron or saline was administered 2 h before IP bacterial challenge with bacterial suspensions of S-plus wild-type *C. fetus* strain 84-32 in normal saline. Iron dextran was dosed to yield final concentrations of elemental iron of 5 and 1 mg, whereas for ferric chloride dosing to achieve 1 and 0.2 mg was done. Five mice were used for each bacterial dilution and for each condition. Mice were observed for death daily for 7 d.

Effect of mouse strain on susceptibility to IP challenge. Because large numbers of viable *C. fetus* 84-32 cells were required to kill the outbred HA/ICR mice, we next examined whether inbred mice with defined genetic characteristics might be more susceptible. For six different inbred strains, susceptibility to IP challenge was not measurably different than for HA/ICR mice (Table III). Previous studies have shown that spleen cells from C3H/HeJ mice do not respond to challenge with bacterial LPS by showing mitogenic or other immune response, whereas the same cells from C3H/HeN mice do respond (24, 25). After parenteral challenge with *Salmonella* species, mortality is higher in the C3H/HeJ mice than in C3H/HeN mice (25). Since *C. fetus* cells contain LPS with long polysaccharide side-chains (26), it seemed likely that after IP challenge C3H/HeJ mice should have a worse outcome than C3H/HeN mice (LPS-responder strain). However, we found in repeated experiments that significantly greater mortality occurred in C3H/HeN (LPS-responder strain) than in C3H/HeJ mice (Table IV) ($P < 0.001$, Yates corrected Chi-squared test).

LD_{50} in outbred mice. In any event, mortality was no greater in C3H/HeN mice than in the outbred HA/ICR mice. Therefore, all subsequent studies were performed in HA/ICR mice to compare virulence between an S-plus strain and its spontaneous S-minus mutant. For these experiments a total of 240 mice were used, 10 for each bacterial dilution for each condition. As expected, the LD_{50} for strain 84-32 (S-plus) was 5.8-fold lower after pretreatment with ferric chloride than after iron dextran (Table V). In both experiments, the LD_{50} for 84-54 (S-minus) was significantly (15.5- and 43.3-fold) greater than for 84-32 (S-plus). At doses $> 10^9$ CFU, both strains produced $\sim$ 100% mortality, whereas differences in mortality were observed at doses $< 10^9$ CFU (Fig. 3). We then examined the time course of mortality after challenge with 3×10^6, 1.5×10^7, 7.5×10^7, 3.8×10^8, and 1.9×10^9 CFU of strains 84-32 and 84-54 with 10 mice at each dose for each strain. The cumulative percent survival from low dose (3×10^6) to high dose (1.9×10^9) for each strain was calculated against time. For both strains, death rarely occurred before 24 h or after 72 h after challenge. Between 24 and 72 h, 29 of 50 mice (58%) infected with strain 84-32 died, compared with 12 of 50 mice (24%) infected with strain 84-54 ($P < 0.001$, Yates corrected Chi-squared test) (Fig. 4). Thus, the virulence of the S-plus

Table II. Comparison of the Mortality of HA/ICR Mice after IP or Intravenous Challenge with C. fetus Strain 84-32

	Percent mortality*	
Bacterial dose/mouse	IP	Intravenous
9.1×10^8	100	60
9.1×10^7	50	0
9.1×10^6	0	0

Each mouse was pretreated with 5 mg elemental iron as iron dextran 2 h before bacterial challenge. 10 mice were used for each bacterial dilution and for each condition.

* Mice were observed for death daily for 7 d.

Table III. Comparison of Susceptibility of Seven Different Mouse Strains to Lethal Infection Due to C. fetus Strain 84-32

	Gene symbols*			Death at dose‡		
Mouse strain	H-2	Ity	LPS	2×10^9	2×10^7	2×10^5
HA/ICR	NA	NA	NA	3/3	0/4	0/4
BALB/c	d	s	n	3/3	0/4	0/4
NZB	d	u	n	3/3	0/4	0/4
B6D2F1	b/d	u	n	3/3	0/4	0/3
C57BL/6J	b	s	n	3/3	0/4	0/4
C3H/HeN	k	r	n	3/3	0/4	0/3
CBA/N	k	r	n	2/3	0/3	0/3

Each mouse was given intraperitoneally 1 ml bacterial suspension containing 5 mg elemental iron as iron dextran. Mice were observed for mortality for 7 d.

* Gene symbols: *H-2*, HMC in mice; *Ity*, gene for immunity to *S. typhimurium* with *r* and *s* as the respective resistant and susceptible alleles (22); *LPS*, gene for responsiveness to toxic and mitogenic effects of LPS with *d* as defective (nonresponder) and *n* as normal (responder) alleles (23); *NA*, not applicable for outbred mouse strain; *u*, unknown.

‡ Number killed/number receiving that dose.

strain was significantly greater than the S-minus strain in this parenteral challenge model.

Oral challenge. Since for humans the presumed route of infection with *C. fetus* is via oral ingestion, we next studied the effects of oral challenge on the HA/ICR mice. After ferric chloride pretreatment, none of 10 mice orally challenged with S-plus strain 84-32 or with its S-minus mutant 84-54 died during a 5-d period. Since in human infections *C. fetus* translocates from the intestinal tract to the bloodstream, we asked whether this phenomenon was related to the presence of the S-protein. Blood cultures from each mouse were obtained before and after oral challenge with strain 84-32 or 84-54. No bacteria were isolated from the blood samples taken before challenge. All five mice challenged with 2×10^8 CFU of 84-32 showed high-grade bacteremia at 30 min, 4 h, 24 h, and 48 h

Table IV. Mortality of C3H/HeN and C3H/HeJ Mice after Challenge with C. fetus Strain 84-32

	Percent death*	
Mouse strains‡	Iron dextran§	Ferric chloride‖
C3H/HeN	72	90
C3H/HeJ	32	40

* Significantly greater mortality occurred in C3H/HeN than in C3H/HeJ mice ($P < 0.001$, Yates corrected Chi-squared test).

‡ C3H/HeN is a LPS^n and Ity^r mouse strain and C3H/HeJ is a LPS^d and Ity^r mouse strain (22, 23).

§ Groups of 25 mice each were pretreated (IP) with 5 mg elemental iron as iron dextran. The cumulative mortality for each mouse strain at doses 10^7 ($n = 5$), 10^8 ($n = 10$), and 10^9 ($n = 10$) CFU of strain 84-32 was calculated.

‖ Groups of 10 mice each were pretreated (IP) with 1 mg elemental iron as ferric chloride and then challenged with 3×10^7 CFU of strain 84-32.

Table V. LD_{50} of C. fetus Strains 84-32 and 84-54 after Iron Pretreatment of Adult HA/ICR Mice

	LD_{50}	
Bacterial strain	Iron dextran	Ferric chloride*
S-plus (84-32)	7.6×10^8	1.3×10^7
S-minus (84-54)	1.2×10^{10}	5.5×10^8
Ratio (84-54/84-32)	15.5	43.3

Groups of 10 mice each were given from 10^5 to 10^{10} CFU of wild-type S-plus *C. fetus* strain 84-32 or its spontaneous mutant S-minus strain 84-54.

* 5 mg elemental iron as iron dextran or 1 mg elemental iron as ferric chloride was administered intraperitoneally 2 h before IP challenge of adult HA/ICR mice with bacterial suspensions of strain 84-32 or 84-54. Mice were observed for death daily for 7 d.

after challenge and became negative between 48 and 120 h. In contrast, *C. fetus* was not isolated from any mice challenged with a similar dose of 84-54 at any time after injection (Table VI). The absence of bacteremia in mice challenged with S-minus strain 84-54 indicated that bacteremia did not result from trauma to the upper gastrointestinal tract during the feeding. To determine whether the presence of the S-protein permitted bacteremia by *C. fetus*, we separately challenged HA/ICR mice with five S-plus and serum-resistant *C. fetus* strains, four S-plus but serum-sensitive strains, or five S-minus and serum-sensitive strains including three wild-type strains and two spontaneous mutants and tested bacteremia 4 h after challenge (Table VII). The nine S-plus strains tested, regardless of serum susceptibility or LPS type, caused bacteremia in 40 of 45 (88.8%) mice 4 h after oral challenge, whereas none of 25 mice challenged with five S-minus strains were bacteremic ($P < 0.001$, Yates corrected chi-square test). We next questioned whether iron pretreatment was necessary for *C. fetus* to cause bacteremia in HA/ICR mice. Bacteremia after challenge with 2×10^7–7×10^8 CFU of S-plus strain 84-32 occurred in 4 of 8 mice pretreated with iron, 5 of 8 mice pretreated with normal saline, and none of 6 mice challenged with S-minus strain 84-54. From these studies we concluded that the presence of an S-protein was associated with bacteremia after oral challenge, regardless of iron pretreatment, serum susceptibility, or LPS type of the challenge strain.

Passive protection of HA/ICR mice from bacteremia after oral challenge. Three groups of seven mice each were challenged orally with 1.1×10^9 ($n = 2$) or 1.5×10^9 ($n = 5$) CFU of

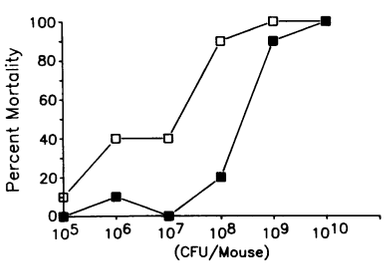

Figure 3. Dose-dependent mortality of mice challenged with *C. fetus* strain 84-32 or 84-54. 10 mice were each given 1 mg iron as ferric chloride intraperitoneally 2 h before challenge with *C. fetus* strain 84-32 (*open squares*) or 84-54 (*solid squares*) and observed for death for 7 d. No difference was found at doses > 10^9 CFU, but at 10^8 CFU S-plus strain 84-32 caused 70% more mortality than its S-minus mutant strain 84-54.

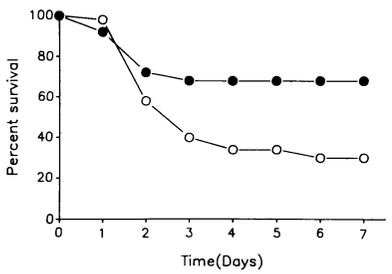

Figure 4. Survival of HA/ICR mice after IP challenge with *C. fetus* S-minus strain 84-54 (*solid circles*) or S-plus strain 84-32 (*open circles*). 50 adult HA/ICR mice were each given 1 mg iron as ferric chloride intraperitoneally 2 h before challenge. Five doses of serial 1:5 dilutions of an inoculum of 2×10^9 CFU of *C. fetus* cells were used for of challenge of 10 mice at each dose for each strain. A cumulative percent survival over time was calculated based on the mortality from the five doses for each strain. Most mortality was observed between 24 and 72 h for both strain 84-32 (58%) and strain 84-54 (24%).

C. fetus strain 84-32. Each mouse was pretreated with 10 μl of rabbit antiserum to 100,000-mol wt purified S-protein, normal rabbit serum, or normal saline as controls. Blood cultures were performed on samples taken at 30 min, 4 h, 12 h, and 24 h after oral challenge. Immune serum to the 100,000-mol wt S-protein was highly protective compared with normal rabbit serum and the normal saline control (Fig. 5). These results support the hypothesis that the S-proteins play an important role in virulence.

Toxicity of S-protein. A group of 10 mice was intraperitoneally challenged with one of two suspensions of strain 84-54, containing either 6.1- or 31.1-fold more S-protein than found on strain 84-32. As expected, IP injection of 4×10^6–4×10^7 CFU of S-plus strain 84-32 caused 80% mortality in the iron-pretreated mice, whereas S-minus strain 84-54 caused none (Table VIII). Suspension of strain 84-54 in a solution containing S-proteins at a 6.1-fold excess over that associated with strain 84-32 caused no mortality, but at a 31.1-fold excess 30% mortality was observed. S-protein alone or strain 84-54 with BSA were administered as controls and were associated with no mortality. To investigate whether the S-proteins are nonspecific virulence factors or only enhance virulence of *C. fetus* strains, we attempted to attach the S-proteins to *Campylobacter jejuni* strain 81-176. In the presence of 5 mM $CaCl_2$, the S-protein readily attached to *C. fetus* strain 84-54 and remained attached as visualized by SDS-PAGE (data not shown) despite three successive washes in Hepes buffer (pH 7.2) with 5 mM $CaCl_2$. In contrast, under the same conditions we could not produce any attachment to the *C. jejuni* strain. As such, we were unable to perform virulence studies with the combination of S-protein and a non-*C. fetus* strain of low virulence. These experiments suggest that *C. fetus* strains may have specific receptors for the S-proteins.

Table VI. Bacteremia in Adult HA/ICR Mice after Oral Challenge with C. fetus

	Log_{10} CFU/ml blood* (mean ± SD)	
Time	S-plus 84-32	S-minus 84-54
h		
0	<1.0±0.0	<1.0±0.0
0.5	4.2±0.2	<1.0±0.0
4	4.1±0.3	<1.0±0.0
24	3.2±0.2	<1.0±0.0
48	4.1±0.6	<1.0±0.0
120	<1.0±0.0	<1.0±0.0

Five mice were used in each group. Each mouse was given 1 mg elemental iron as ferric chloride 2 h before oral challenge with 1.9×10^8 CFU of strain 84-32 or with 2×10^8 CFU of strain 84-54.
* Sensitivity of assay: blood culture detects 10 bacterial CFU/ml blood. Between 0.5 and 48 h after challenge with S-plus strain 84-32 each mouse sampled was bacteremic at times, whereas none of the mice challenged with S-minus strain 84-54 were bacteremic at any time.

Discussion

The results of this study demonstrate a correlation between the presence of S-proteins and virulence of *C. fetus* strains. A strain encapsulated with S-proteins has a lower LD_{50} value for IP challenge in mice than its unencapsulated mutant strain, and encapsulated strains are able to penetrate the gastrointestinal mucosa after oral challenge, causing bacteremia and systemic infection.

The IP challenge model could be considered as artificial because of iron pretreatment and high dose of *C. fetus*. However, *C. fetus* is an opportunistic pathogen of humans. Most hosts with systemic *C. fetus* infections are compromised by liver disease, neoplasia, or immunosuppression (7); iron pretreatment serves an analogous function in the experimental model. Similar to humans, normal mice are resistant to *C. fetus*, even at doses as high as 10^{10} CFU, but become susceptible after compromise (in this case, iron pretreatment). High dose is necessary because this organism is an opportunistic pathogen that is not as virulent as *Salmonella typhi* to humans or *Salmonella typhimurium* to mice. The IP challenge model resembles an endotoxemia model. However, it is a infection model as well, despite lack of direct evidence of replication of organisms in the peritoneal cavity and spread to the reticuloendothelial system. In earlier work we showed a significantly greater increase in splenic size after IP challenge with the S-plus strain than the S-minus strain (Blaser, M. J., and D. J. Duncan, unpublished data). In our experiment to protect mice from lethal IP challenge with S-plus strain 84-32, 70% of mice were protected with antiserum to S-protein compared with 30% protected with antiserum to the heat-stable O antigen (mostly LPS) of same bacteria (Pei, Z., and M. J. Blaser, unpublished data). In this model, most mice died between 24 and 72 h after challenge, similar to that observed with endotoxemia. A typical endotoxemia model would not differentiate an S-plus strain from an S-minus strain when mice are challenged with the same doses of bacteria and thus LPS (26); however, the *C. fetus* IP challenge model that we used clearly did so.

Iron is essential for the growth of essentially all bacteria (27, 28), and iron overload appears to be a risk factor for systemic *C. fetus* infection in humans (1–4). The growth of pathogenic bacteria in vivo depends on their ability to obtain iron bound by host substances, such as transferrin and lactoferrin (29–32). Most aerobic bacteria produce siderophores, high-affinity compounds that bind, solubilize, and transport iron across the cell membrane (33, 34), but none are produced by *Campylobacter* species (35). Iron-containing compounds such as mucin, iron dextran, and ferric chloride have been

Table VII. Relationship of Presence of S-proteins and Induction of Bacteremia after Oral Challenge of HA/ICR mice with C. fetus

Strain	Source	Major S-protein	LPS type*	Serum killing‡	Challenge dose	Bacteremia‖
84-32	Bovine vagina	100,000	A	<0.05	2×10^8	5/5
82-40LP	Human blood	100,000	A	<0.05	1×10^9	5/5
84-86	Human blood	100,000	A	<0.05	4×10^7	3/5
80-109	Human blood	127,000	A	<0.05	1×10^8	5/5
84-112	Bovine genital	149,000	A	0.06	1×10^6	4/5
84-91	Human blood	100,000	AB	2.96	2×10^9	5/5
84-94	Human blood	127,000	AB	2.70	1×10^8	3/5
84-107	Human blood	100,000	B	2.71	5×10^8	5/5
84-108	Human blood	100,000	B	2.56	5×10^8	5/5
81-170	Bovine	—	B	2.50	2×10^8	0/5
83-88	Human blood	—	B	1.63	1×10^8	0/5
84-90	Bovine fetus	—	B	3.93	8×10^8	0/5
84-54	Laboratory mutant	—	A	2.10	2×10^8	0/5
82-40HP	Laboratory mutant	—	A	1.02	4×10^9	0/5

Each mouse was pretreated with 1 mg elemental iron as ferric chloride IP 2 h before oral challenge and checked for bacteremia 4 h after challenge. * LPS serotype determined as described (7, 12). ‡ Serum killing is expressed in Log_{10}. Data are from reference 7. ‖ Number with bacteremia detected/number receiving that dose.

used to enhance the virulence of *Neisseria* (20), *Vibrio* (21), *Campylobacter* (19), and other species. In the present study, the virulence of *C. fetus* strains was enhanced at least 100-fold by pretreatment of mice with iron dextran or ferric chloride. Although the mechanisms by which iron compounds increase susceptibility to *C. fetus* infection were not determined, saturation of transferrin, or impaired murine macrophage function may have been factors (36–39).

Several lines of evidence suggest that the *C. fetus* S-proteins are not toxic per se but enhance virulence when present on bacterial cell surfaces as a capsule. (*a*) The LD_{50} for encapsulated strain 84-32 is significantly lower than its unencapsulated mutant 84-54. (*b*) After oral challenge, encapsulated strains are able to cause bacteremia, while unencapsulated strains are not, regardless of serum susceptibility. (*c*) High concentrations of free S-protein mixed with an unencapsulated strain enhance its virulence. (*d*) other studies in our laboratory have shown that removal of the S-protein coat from the surface of strain 84-32 using pronase results in a 40-fold LD_{50} increase, similar to that caused by the S-protein–deficient mutant 84-54 (40). (*e*) Finally, the evidence that these S-proteins are virulence determinants was further strengthened by the passive protection of HA/ICR mice with antiserum to S-protein from high-degree bacteremia after oral challenge. Our previous in vitro work indicates that one of the mechanisms by which the S-protein capsule protects *C. fetus* is via its inhibition of C3b binding, preventing immune elimination (11).

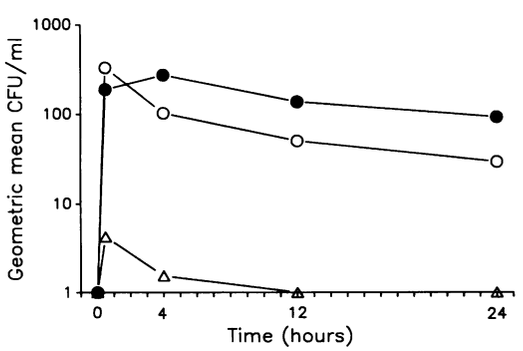

Figure 5. Passive protection of HA/ICR mice from bacteriemia after oral challenge. Three groups of seven mice each were passively immunized with 10 μl of antiserum to 100,000-mol wt S-protein (*open triangles*), normal rabbit serum (*solid circles*), or normal saline (*open circles*) 4 h before oral challenge with 1.1×10^9 ($n = 2$) or 1.5×10^9 ($n = 5$) CFU of S-plus strain 84-32. Blood cultures were performed before or at 0.5, 4, 12, and 24 h after challenge. Geometric mean colony-forming units/milliliter of blood was plotted against time. Bacteremia in mice pretreated with anti-S-protein serum was significantly lower than in controls.

Oral challenge of mice with *C. fetus* provides a relevant model for study of human infection. The ability to cause bacteremia after oral challenge was related only to the presence of S-proteins, not to iron pretreatment, LPS type, or the susceptibility of *C. fetus* strains to killing by normal serum. S-plus *C. fetus* strains can apparently penetrate the intestinal wall and produce bacteremia lasting 48–120 h after oral challenge. The mechanism for this penetration has not been determined, but it is clearly rapid in onset. Although no illness or mortality was observed as a result of bacteremia, we might expect longer-lasting bacteremia, morbidity, and even mortality in orally challenged immunocompromised mice, similar to that observed in compromised humans (1, 3, 4).

Immunocompromise is a prerequisite for *C. fetus* to cause systemic infection in humans (1, 3, 4), suggesting that immune function is highly related to host susceptibility to *C. fetus* infection. Most S-plus strains are resistant to in vitro killing by normal serum, but exceptions have been noted (7, 8). Such strains are encapsulated with S-proteins but have type B LPS instead of the type A LPS usually present in serum-resistant strains. Comparing these strains with S-plus strains containing type A LPS, we expected to find that the serum-resistant strains would cause bacteremia, whereas the serum-sensitive strains would not. However, our results clearly showed that

Table VIII. Effect of Adding S-proteins to S-minus C. fetus Cells in Relation to Virulence after Parenteral Challenge

Strain	No. of mice challenged	Treatment*	Dose	Mortality‡
			CFU/mouse	%
84-32	20	None	4.0×10^6–4.0×10^7	85
84-54	20	None	9.1×10^6–9.1×10^7	0
84-54	20	BSA (0.9–25 μg)	8.0×10^6–8.0×10^7	0
84-54	10	S-protein (0.9–9 μg)	1.3×10^7–1.3×10^8	0
84-54	10	S-protein (25 μg)	7×10^7	30
None	7	S-protein (25 μg)	—	0

Mice were pretreated with 1 mg elemental iron as iron dextran 2 h before challenge with bacteria. * Treatment was performed by dilution of S-minus strain 84-54 in buffer containing S-proteins from strain 84-32 at 6.1-fold (0.9–9 μg/mouse) or 31.1-fold (25 μg/mouse) more than usually are complexed to strain 84-32 as determined by S-protein/cell ratio (see Methods). Strain 84-54 was suspended in equivalent amounts of BSA as control. The high dose (25 μg) of S-protein alone was administered to determine whether it was independently toxic. ‡ Mice were observed for death daily for 7 d.

encapsulated *C. fetus* strains caused bacteremia after oral challenge, regardless of serum sensitivity. Thus, serum susceptibility may be an imperfect marker for virulence of *C. fetus* strains. The S-plus *C. fetus* strains with type B LPS may have a virulence mechanism different from that of the type A LPS strains.

To find mouse strains more susceptible to *C. fetus* infection, we compared an outbred and six inbred mouse strains with different H-2, Ity, and LPS-response genes. Susceptibility of mice to *C. fetus* infection was independent of H-2 and Ity genes. However, by comparing paired LPS^d mice (C3H/HeJ) and LPS^n mice (C3H/HeN), we found that susceptibility was linked to LPS responsiveness, which is independent of Ity status (22). C3H/HeJ mice are defective in their response to most biological effects of LPS (41–45) due to a mutation at the LPS gene locus on chromosome 4 (25). The LD_{50} of *S. typhimurium* is < 2 CFU in LPS^d C3H/HeJ mice and 10^3–10^4 CFU in LPS^n C3H/HeN mice (22). In contrast to these observations, greater mortality was found in LPS^n C3H/HeN mice challenged with S-plus *C. fetus* than in LPS^d C3H/HeJ mice (Table IV). The LPS gene controls both susceptibility to the toxic effects of LPS and specific and more generalized immunoregulatory responses to LPS (25, 41–45). Defects in the LPS gene of C3H/HeJ mice render them resistant to endotoxic shock after challenge with purified LPS at concentrations that are lethal to other C3H lineages and unresponsive to the immunostimulatory effects of LPS (25, 41–45). *C. fetus* is a pathogen adapted to the bovine and ovine species, and is clearly much less virulent in mice than *S. typhimurium* with significantly higher LD_{50}s even for the more virulent S-plus strain. However, *C. fetus* LPS has endotoxic activity similar to that of the *Enterobacteriaceae* (26). As evidenced by the temporal characteristics of *C. fetus*–induced lethality, the toxic effects of LPS may be important virulence factors, whereas LPS at the challenge level may have minimal toxic effects in *S. typhimurium*–infected mice. In the present study, relative resistance of C3H/HeJ mice to the toxic effects of LPS is the most likely factor responsible for their lower susceptibility than C3H/HeN mice to *C. fetus* infection.

In conclusion, we have described a mouse model of *C. fetus* infection that is relevant to human infection. The presence of S-proteins is an important virulence factor after parenteral or oral challenge of these animals. These in vivo studies raise a number of interesting questions relating to the mechanisms by which encapsulated *C. fetus* strains cause disease, such as the role of iron in *C. fetus* infection, differences between S-proteins from *C. fetus* strains with type A LPS or type B LPS, and how S-proteins enable *C. fetus* to penetrate the intestinal wall and cause systemic infection.

Acknowledgments

This work was supported in part by the U. S. Army Medical Research and Development Command, by the Medical Research Service of the Veterans Administration, and by the National Institute of Allergy and Infectious Disease (R01 AI-24145-01A1). Dr. Blaser is a Clinical Investigator of the Veterans Administration.

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