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Murine Intranasal Challenge Model for the Study of *Campylobacter* Pathogenesis and Immunity

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Campylobacter jejuni* infection of mice initiated by intranasal administration was investigated as a potential model for studies of pathogenesis and immunity. By using a standard challenge (5×10^9 CFU), *C. jejuni* 81-176 was more virulent for BALB/c (72% mortality) than for C3H/Hej (50%), CBA/CAJ (30%), or C58/J (0%). Intranasal challenge of BALB/c was used to compare the relative virulence of three reference strains; *C. jejuni* 81-176 was more virulent (killing 83% of challenged mice) than *C. jejuni* HC (0%) or *C. coli* VC-167 (0%). The course of intranasally initiated *C. jejuni* 81-176 infection in BALB/c was determined. *C. jejuni* was recovered from the lungs, intestinal tract, liver, and spleen at 4 h after challenge, the first interval evaluated. After this initial interval, three distinct patterns of infection were recognized: (i) a progressive decline in number of *C. jejuni* CFU (stomach, blood, lungs), (ii) decline followed by a second peak in the number of organisms recovered at 2 or 3 days postchallenge (intestine, liver, mesenteric lymph nodes), and (iii) persistence of approximately the same number of *C. jejuni* CFU during the course of the experiment (spleen). Intranasally induced infection initiated with a sublethal number of bacteria or intranasal immunization with killed *Campylobacter* preparations resulted in both the generation of *Campylobacter* antigen-specific immune responses and an acquired resistance to homologous rechallenge. The model was used to evaluate the relative virulence of nine low-in vitro-passage (no more than five passages) isolates of *C. jejuni* species from patients with diarrhea. The patient isolates were differentially virulent for mice; one killed all exposed mice, three were avirulent (no deaths) and the remainder showed an intermediate virulence, killing 17 to 33%. Mouse virulence of *Campylobacter* strains showed a trend toward isolates originating from individuals with watery diarrhea; however, no association was found between mouse virulence and other signs or symptoms. There were no observed relationships between mouse virulence and bacterial Lior serotype or Fla polymorphic group. Intranasal challenge of BALB/c with *C. jejuni* is a useful model for the study of infection and vaccination-acquired immunity to this agent.

Campylobacter jejuni is a major cause of gastroenteritis (36, 38) and enterocolitis in humans. Infection may be asymptomatic or associated with disease ranging in severity from acute watery diarrhea (13) to inflammatory disease that may be associated with a tissue invasion (26) and at times with bacteremia (19). Immunity to *Campylobacter* disease can develop as the result of repeated infections (11, 13, 36), and, thus, the feasibility of a vaccine for prevention of *Campylobacter* disease is suggested.

The mechanism(s) of immune protection to *Campylobacter* infection and disease is poorly understood, and thus little information is available to guide vaccine development. In part, this deficit in knowledge results from the lack of an animal model of *Campylobacter* infection in which high-resolution immunologic measurements are possible. Only humans (11), young *Rhesus* monkeys (17, 33) and weaning or young ferrets (9, 18) are known to be naturally susceptible to *C. jejuni*-caused diarrheal disease. The removable intestinal tie adult rabbit diarrhea (RITARD) model (15), oral feeding of *Campylobacter* organisms to adult mice (6, 12), mice pretreated with iron (35), and challenge of infant mice (1) have been used for selected studies of pathogenesis and immunity. However, with the exceptions of ferrets and infant mice, none of the small-animal models exhibit diarrhea associated with infection. Infant mice provide very limited platforms for immunologic studies because of their size and immunologic immaturity, and the ferret model has limited use due to a lack of availability of immunologic reagents.

Investigators attempting to develop and evaluate *Shigella* vaccines have had to address similar issues with animal models. For *Shigella* strains, it was found that intranasal challenge of mice at times resulted in disease and when disease occurred the responsible strain was of a phenotype that associated with virulence for human intestinal disease (28, 39). The *Shigella* mouse intranasal challenge model also proved useful for studies of immunity to this agent, as well as in evaluating relative reactogenicity and efficacy of live attenuated candidate vaccines (28). We report here studies which show that intranasal challenge of mice is a useful model for the studies of *Campylobacter* pathogenesis and immunity.

MATERIALS AND METHODS

Mice. BALB/c, C3H/HeJ, CBA/CAJ, and C58/J mice (age 6 to 8 weeks) were purchased from Jackson Laboratory (Bar Harbor, Maine) and housed in laminar flow cages for a minimum of 8 days before being used for experiments. Standard laboratory animal chow and water were provided ad libitum. Research met the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services Publication (National Institutes of Health) 86-23, 1985 edition.

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***Campylobacter* strains.** All *Campylobacter* strains were grown at 42°C in an atmosphere of 85% N_2, 10% CO_2, and 5% O_2 in media described below. The source and passage history of the reference strains, *C. jejuni* 81-176 (11), *C. jejuni* HC (8, 15), and *C. coli* VC-167 (32) are reported in detail elsewhere. For the present studies strain 81-176 was grown on trypticase soy agar supplemented with sheep blood (TSAB); the resulting lawn was suspended in phosphate-buffered saline (PBS), and 5×10^8 CFU were inoculated intravenously into BALB/c mice. After 3 days, the mice were sacrificed and their spleens were removed and homogenized in PBS. The resulting suspension was used to infect additional mice by the intravenous route. After three passages, spleen homogenate was plated on TSAB and the resulting bacteria were suspended in 15% glycerol-brain heart infusion (GBHI) broth (Difco Laboratories, Detroit, Mich.) and stored at −70°C as a primary seed. Strains HC and VC-167, from the culture collection of the Naval Medical Research Institute, Bethesda, Md. (8, 32), were plated on TSAB, and the resulting bacteria were suspended in GBHI and frozen as primary seeds.

Nine isolates of *C. jejuni* were from a collection of cryopreserved bacteria isolated from patients who presented with diarrhea to a primary health-care clinic in the Abees village complex, Alexandria Governorate, Egypt, between 11 December 1991 and 11 August 1992. These patients (group mean age, 7.1 years; standard deviation, 11.2 years; range, 0.4 to 35.0 years; mode, 0.4 years; three females) were informed of the purpose of the study and consented to participate (a caregiver completed informed consent for children). An interview of the patient (or a caregiver) was used to elicit age; subjective reports of fever, duration of the current episode of fever, if present; duration of the present episode of diarrhea; and whether or not there was blood in stools. Stool samples were collected from each patient and examined for bacterial and parasitic agents (20, 23) known to be capable of causing diarrhea. Also in the laboratory, stools were classified on form ([classifications: formed [normal], softer than normal [listed as soft in Table 2], or shape of the container [listed as watery in Table 2]) and the presence of blood (gross blood and/or guaiac test positive), leukocytes (20) (classified categorically as 0, 1 to 5, 6 to 25, or >25 per high-powered field [×100 objective and ×10 ocular lens]), and mucus (classified as normal or increased). Stools were recorded as blood positive if a patient, caregiver, or laboratory technician reported seeing blood or if the guaiac test was positive.

Campylobacter strains from Egyptian patients were obtained from a clinical laboratory that used standard procedures for isolation and/or identification of bacterial and parasitic agents (23). Lior serotyping (24) and Fla genotyping (3, 29) for each isolate were done by established procedures. Each isolate was then passed on a TSAB plate. The resulting bacterial lawn was harvested and prepared for storage in liquid nitrogen by using the Micro-bead system (Pro-Lab Diagnostics, Austin, Tex.). Frozen cultures were transported between laboratories in vapor phase nitrogen transporters.

To limit the potential for phenotypic or genetic changes in strains, each isolate was used at a fixed passage level. This was accomplished by preparing primary and secondary seeds from each. Each Egypt isolate was inoculated onto TSAB, and the resulting bacteria were suspended and frozen as a primary seed by using Micro-bead reagents and methods. The primary seeds of all bacterial isolates were separately inoculated onto TSAB, and the resulting lawn was suspended and then frozen by using 15% glycerol or Micro-bead as secondary seeds. Thus, the Egyptian *Campylobacter* isolates used in this study were passaged in vitro not more than five times before use. Only campylobacters isolated from stools that did not yield other bacterial or parasitic diarrheagenic microbial agents were selected for this study.

To prepare bacterial suspensions for infecting mice, secondary seeds were grown on TSAB for 18 h and then inoculated into a biphasic culture of brain heart infusion (BHI) agar and broth supplemented with 1% yeast extract (YE) and incubated for an additional 18 to 20 h (6). The broth phases of these cultures were separately collected and centrifuged at 8,000 × *g* at 4°C for 20 min and suspended in spent medium to 1/20 of their original volume (working culture). The number of *Campylobacter* isolates in working cultures was estimated by spectrophotometric determinations (6, 8), and dilutions based on these estimates were used to challenge mice. Challenge doses were more exactly determined after challenge by plating serial dilutions of each working culture on TSAB plates.

Intranasal inoculation. Mice were lightly anaesthetized with methoxyflurane (Metofane, Pitman-Moore, Inc., Mundelein, Ill.). Using a Gilson micro-pipetman (Rainin Instruments, Emeryville, Calif.), 30 μl of the selected bacterial suspension or vaccines (see below) was then applied a drop at a time (5 to 6 μl at a time) to the external nares. Control mice were similarly inoculated with 30 μl of BHI-YE medium or PBS.

Illness index. An index was developed to allow quantitative expression of the relative degree of morbidity and mortality associated with intranasal infection. This was accomplished by observing infected mice once per day for 6 consecutive days following challenge, beginning at 24 h after infection. At each observation interval, each mouse was assigned a score of 0, 1, or 2 denoting apparently healthy, ill (a hunched back, ruffled fur, lethargic), or dead, respectively. For each observation day, the total score within each group was divided by the number of mice observed to yield the day index. The mean of these daily indices is presented as the illness index for the each group reported in the result section.

Course of *C. jejuni* infection. At intervals after infection with 10^9 CFU of 81-176, blood was collected by cardiac puncture and various organs and tissues were collected from each of 4 or 5 randomly selected mice. Organs were homogenized individually; lungs, stomach, and spleen in 1 ml; large intestine, small intestine, and liver in 5 ml; and mesenteric lymph nodes in 0.5 ml of cold PBS. Dilutions of each homogenate were plated in duplicate onto TSAB supplemented with cefoperazone, vancomycin, and amphotericin B (CVA) (Remel Microbiology Products, Lenexa, Kans.). After 48 h of incubation, *C. jejuni* colonies were counted. Data are expressed as $\log_{10}$ CFU per organ or tissue or milliliter of peripheral blood.

Vaccines. Prototype killed whole-cell vaccines were prepared from the 81-176 strain (6, 7). The vaccines evaluated comprised heat-killed (60°C, 60 min) (HK) or formalin-fixed (0.025M) (FF) *Campylobacter* whole cells or a 1:1 mixture (HK:FF) of these preparations.

Measurement of antibodies. Intestinal lavage fluid was collected (7 days after vaccination) in EDTA (50 mM)-phenylmethylsulfonyl fluoride (100 mM) solution by orally feeding four doses of buffered polyethylene glycol (50 mM) followed by intraperitoneal inoculation of 100 μg of pilocarpine (6, 8). After dispersing solid materials and centrifugation (4°C, 3,000 × *g*, 20 min), supernatant was collected and the protein concentration was determined and adjusted to 2 mg/ml with PBS. Samples were stored in aliquots at −70°C.

Whole blood (0.1 ml) was collected (21 days after vaccination) from snipped tails into 0.9 ml of PBS containing 0.1% Tween 20. After 1 cycle of freezing and thawing, samples were centrifuged at 400 × *g* at 4°C for 15 min and supernatant fluids were used for antibody determinations.

A glycine extract (25) of outer membrane proteins from strain 81-176 was used (3 μg/ml) as an antigen to detect *Campylobacter*-specific immunoglobulin A (IgA) and IgG by established enzyme-linked immunosorbent assay procedures (6, 8). Endpoint titers were expressed as the reciprocal of the highest dilution giving an absorbance value greater than 2 standard deviations above background. Background was determined by using lavage fluid (optical density = 0.14) or lysed blood (optical density = 0.15) from mice that had no known exposure to *Campylobacter* species.

Isolation of mononuclear cells from tissues. Selected organs and tissues were removed from mice by sterile procedures and rinsed thoroughly in Hanks balanced salt solution. Spleen and lungs were then teased apart by using forceps, and mononuclear cells were isolated from the resulting suspensions by Ficoll density gradient centrifugation (7). Mesenteric lymph node cells were isolated by dissecting fat from the nodes and then pressing the nodes lightly with the flat portion of the glass bottle stopper. Lamina propria and Peyer's patch cells were isolated by collagenase digestion (21). Isolated cells were washed two times in RPMI containing 10% fetal calf serum, 2 mM glutamine, and 50 μg of gentamicin per ml and suspended at a concentration of 5×10^6 cells per ml.

Assay for antibody secreting cells. *Campylobacter*-specific IgA- or IgG-secreting cells were determined by using a slight modification of previously published methods (7, 30). Plates were coated with glycine extract antigen (25) prepared as described above. Mononuclear cells were incubated on antigen-coated plates for 14 to 16 h at 37°C in a 5% CO_2 environment followed by an additional 2 h of incubation at 37°C after addition of alkaline phosphate-conjugated goat anti-mouse immunoglobulins. Spots were developed by using NBT-BCIP (Nitro Blue Tetrazolium, 5-bromo-4-chloro-3-indolylphosphate) agar substrate (3 to 4 h at 4°C) and counted by using a dissecting microscope at ×30 magnification.

Fecal excretion. Fecal excretion of *Campylobacter* spp. was monitored daily for 9 consecutive days after challenge by culturing fecal homogenates (approximately 5% suspension in PBS) on CVA plates. After 48 h of incubation, plates were examined for the presence of *Campylobacter* colonies. Colonies were confirmed as *C. jejuni* or *C. coli* by morphology, catalase, and oxidase reactions.

Vaccine efficacy. Efficacy was determined 4 weeks after immunization by intranasally challenging mice with approximately 5×10^9 CFU of *C. jejuni* (81-176). Measures of efficacy were illness index, mortality and the presence and duration of intestinal colonization. Efficacy was calculated as (rate for control mice − rate for vaccinated mice / rate for control mice) × 100.

Statistical analysis. Comparisons of rates was by χ^2 or Fisher exact test, and comparisons of means employed the Student *t* test. *P* values of ≤0.05 were considered significant.

RESULTS

Effect of mouse strain. Four strains were challenged intranasally with decreasing doses of *C. jejuni* 81-176. The outcome was measured as cumulative mortality at day 6 after infection (Fig. 1). Two patterns of susceptibility were noted: relatively resistant (C58/J) and more susceptible (BALB/c, C3H/HeJ, CBA/CAJ). BALB/c mice were selected for subsequent experiments.

Effect of *Campylobacter* strain or species. Separate groups of mice were challenged with *C. jejuni* 81-176, *C. jejuni* HC, or *C. coli* VC-167. Outcome variables were cumulative mortality,

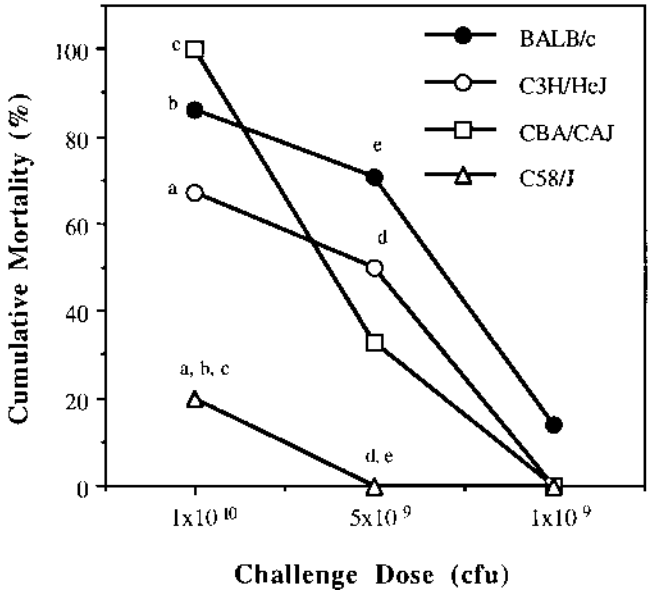

FIG. 1. Susceptibilities of selected strains of mice to intranasal challenge with *C. jejuni* 81-176. Fourteen BALB/c, 12 C3H/HeJ, 12 CBA/CAJ, and 10 C58/J mice were infected with the indicated doses, and the cumulative mortalities at 6 days following challenge are presented. At each challenge dose, points marked with the same letter denote pairs that differ significantly ($P < 0.05$).

occurrence of bacteremia, and duration of excretion of the challenge strain in feces.

Virulence varied as a function of bacterial dose, strain, and species (Table 1); *C. jejuni* 81-176 was the most virulent, and *C. coli* was the least virulent. The three signs of infection used as outcome variables showed concordant results as did the derived illness index. Animals challenged with *C. jejuni* 81-176 remained colonized for the longest interval followed by *C. jejuni* HC, whereas *C. coli* caused only transient colonization.

Strain 81-176 was selected for the following experiments.

Course of infection. After intranasal inoculation of 10^9 CFU, three patterns of infection were seen (Fig. 2). In the lungs, blood, and stomach, peak numbers of *C. jejuni* were found at the first interval evaluated, 4 h after infection. After that, the bacterial burden decreased, reaching undetectable levels by day 2 for the blood and stomach and day 7 for the lungs.

A second pattern was found for the large and small intestines, liver, and mesenteric lymph nodes. At these sites infection was biphasic. Substantial *C. jejuni* were present at 4 h, decreased through 2 or 3 days postinfection, and then rebounded with a second peak at 4 days. In all instances when second peaks were demonstrated, substantial numbers of *C. jejuni* persisted through day 7, at which time experiments were terminated.

The third pattern was seen only in the spleen. In this organ,

TABLE 1. Susceptibility of BALB/c mice to intranasal infection with selected *Campylobacter* strains[a]

Strain and dose	*n*	% Dead	Illness index	Fecal excretion	% Bacteremia
C. coli VC167					
5×10^9	8	0[a]	0.2 (0.4)[c]	3.5 (1.1)[f,g]	0[i]
5×10^8	8	0	0[d]	2.0 (1.1)[h]	0
C. jejuni HC					
5×10^9	8	0[b]	0.4 (0.5)	5.1 (1.8)[f]	25[j]
5×10^8	6	0	0.1 (0.2)[e]	3.3 (2.1)	0
C. jejuni 81-176					
5×10^9	12	83[a,b]	0.9 (0.4)[c]	9.0 (0)[b,g]	75[i,j]
5×10^8	12	17	0.6 (0.4)[d,e]	6.9 (1.0)[h]	33

[a] Mice were challenged with the indicated *Campylobacter* strain or species at day zero and were monitored for the next 6 days for mortality and illness and for the next 9 days for fecal excretion. Data are mean illness indexes (standard deviations) (see Materials and Methods) or mean days of fecal excretion (standard deviations). Bacteremia was determined from samples collected at 16 h after infection. Within each column, points marked with the same roman superscript letter differ at $P \leq 0.05$.

[b] Data on colonization were obtained from two surviving animals.

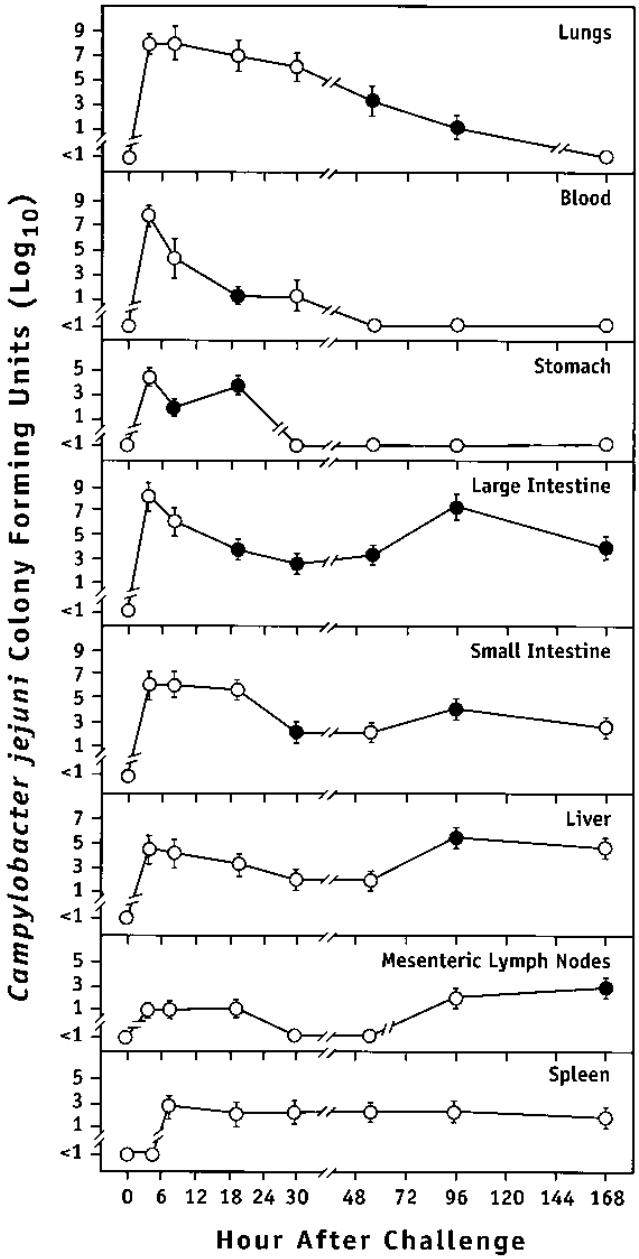

FIG. 2. Course of *C. jejuni* 81-176 infection in BALB/c mice. Mice were infected intranasally with 10^9 CFU, and at each indicated interval 4 or 5 were sacrificed and the number of *C. jejuni* in peripheral blood and selected tissues and organs was determined. Data are expressed as means of $\log_{10}$ CFU per organ or per ml of blood. Solid symbols denote a statistically significant difference ($P < 0.05$) with the preceding time point.

TABLE 2. Virulence for BALB/c mice of low-passage clinical isolates of *C. jejuni* and relationships with mouse virulence, selected patient signs and symptoms, and selected measures of bacterial phenotype/genotype

Isolate no.[a]	Mouse virulence[b]		Patient signs and symptoms						No. with isolate phenotype		
			Stool[c]				No. of days of[c]				
	% Dead	Illness index[c]	Form	Mucus	Blood	No. of leukocytes	Diarrhea	Fever	Lior	Fla A	Fla B
749	100	1.5 (0.7)	Watery	Increased	No	1–5	4	2	36	8	5
715	33	0.8 (0.5)	Watery	Increased	No	1–5	4	0	105	13	13
756	17	0.9 (0.4)	Watery	Increased	No	6–25	7	0	18	10	10
547	17	0.8 (0.4)	Watery	Normal	No	1–5	2	3	1	5	5
616	17	0.7 (0.5)	Watery	Normal	No	1–5	20	0	1	7	7
617	17	0.5 (0.5)	Soft	Normal	No	1–5	7	0	2	13	13
755	0	0.3 (0.5)	Soft	Increased	Yes	1–5	2	0	55	13	13
1387	0	0.3 (0.5)	Soft	Increased	Yes	6–25	5	5	36	10	10
555	0	0.3 (0.5)	Soft	Normal	No	>25	2	0	2	ND[d]	ND[d]

[a] The numbers presented are the United States Naval Medical Research Unit No. 3 culture collection identifiers with the prefix ABEES deleted.
[b] Six mice were challenged with each isolate.
[c] Detailed definitions of these measures can be found in Materials and Methods.
[d] ND, not done.

C. jejuni persisted at essentially unchanged numbers through day 7.

Mouse virulence of patient isolates. Patient isolates were differentially virulent in the intranasal model; one isolate was lethal for all exposed mice, five caused mortality in 17 to 33%, and three were essentially avirulent (Table 2). The campylobacters with higher mouse virulence showed a tendency toward association with patients who had watery stools. There was no obvious association between mouse virulence and any indicators of invasive diarrhea (fever, blood, mucus) or with the measured bacterial phenotypes (Lior serotype, Fla polymorphism).

Studies of acquired immunity. (i) Infection-induced resistance. An experiment was done to learn if immunization by infection with a sublethal dose of 81-176 (5×10^7 CFU) would induce an immune response and resistance to a second homologous challenge with a high dose of 81-176. The immunizing infection induced both secretory IgA (sIgA) (endpoint titer, 187 ± 109) and serum antibodies (IgA, 1,530 ± 991; IgG, 12,150 ± 2,662) which reacted with *C. jejuni* antigens. When protection was measured as illness index or intestinal colonization, it was found that primary infection afforded 70 to 91% resistance from homologous challenge (4×10^9 CFU; Table 3).

(ii) Vaccine-induced resistance. To determine if immunization with inactivated *C. jejuni* might protect from subsequent *C. jejuni* challenge, separate groups were immunized intranasally with three doses (at 48-h intervals) of HK, FF, or HK:FF vaccines, each containing 2×10^7 bacterial cells in 30 μl of PBS. Vaccination did not cause observable adverse effects.

All formulations induced similar levels of serum IgA; the serum IgG response following FF vaccine was significantly less ($P < 0.05$) than that induced by the other vaccines (Fig. 3). HK and FF alone induced similar levels of sIgA. Compared to the response seen in HK-immunized animals, the mixture of the two vaccines induced significantly ($P > 0.05$) higher levels of sIgA.

TABLE 3. Resistance to *Campylobacter jejuni* following infection or vaccination[a]

Immunization	Immunogen	*n*	Measurements of immunization efficacy[b]			
			Illness		Fecal excretion	
			Index[2]	% Efficacy	% Colonized	% Efficacy
Infection	81-176	12	0.27 (0.39)^{a}	71^{e}	8^{i}	91^{m}
Control	BHI-YE	12	0.92 (0.30)^{a}	0^{e}	90^{i}	0^{m}
Vaccine	HK	6	0.44 (0.27)^{b}	67^{f}	0^{j}	100^{n}
	FF	6	0.39 (0.28)^{c}	83^{g}	0^{k}	100^{o}
	HK:FF	6	0.31 (0.31)^{d}	100^{h}	17^{l}	83^{p}
Control	PBS	8	1.05 (0.51)^{b,c,d}	0^{f,g,h}	100^{j,k,l}	0^{n,o,p}

[a] Mice were immunized with three doses of 2×10^7 vaccine particles or infected with 5×10^7 CFU of bacteria. Six weeks later they were challenged intranasally with 4×10^9 CFU of *C. jejuni* 81-176 and observed for the next 6 days for illness and death. Fecal excretion of the surviving mice was determined at day 7. Derivation of illness indices and procedures for vaccine efficacy calculations are presented in Materials and Methods.
[b] Within each vertical data column, points marked with the same roman superscript letter differ at $P \leq 0.05$.

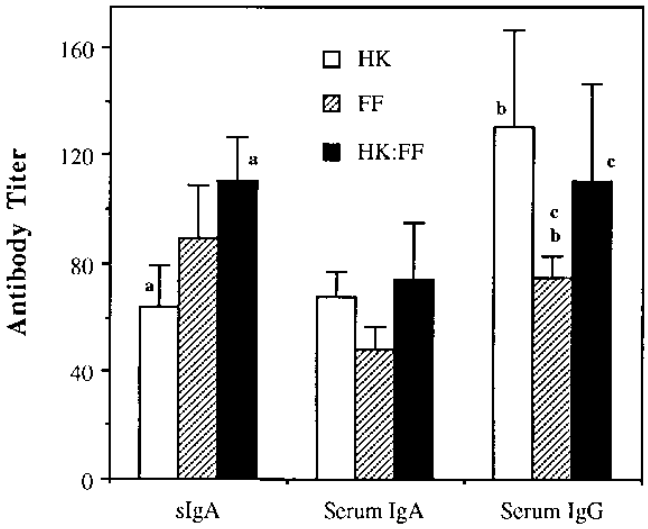

FIG. 3. Secretory and systemic antibody responses after vaccination. Separate groups of six mice were immunized with three doses of one of the indicated vaccines intranasally at 48-h intervals. Intestinal lavage fluid (day 7) and blood samples (day 21) were collected after immunization, and the levels of *Campylobacter*-specific antibodies were determined by using *C. jejuni* 81-176 glycine extract as antigen. Data are presented as mean antibody titers and standard deviations; bars marked with the same letter are significantly different ($P < 0.05$).

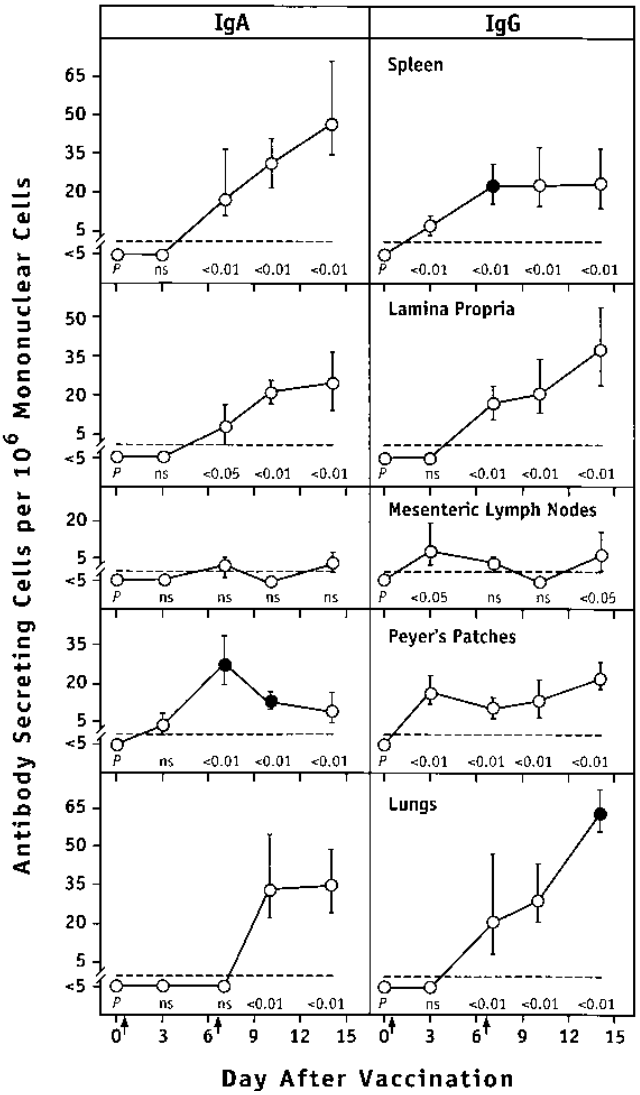

FIG. 4. Dynamics of *Campylobacter*-specific ASC responses after vaccination. Mice were immunized intranasally with two doses of formalin-fixed antigen. At each time point, five mice were sacrificed and mononuclear cells were separated from the indicated tissues and organs. The number of *Campylobacter*-specific ASC/10^6 mononuclear cells was determined for each mouse. The mean and standard error of the mean of the natural logs of these values were determined for each isotype at each time point. The figure presents these mean values after back-transformation to linear form. The dashed line represents the detection limit of the assay. Also presented are *P* values (χ^2) for comparisons of frequency of responders (responder = ≥5 *Campylobacter*-specific ASC/10^6 mononuclear cells) at time points of >0 days with frequency of responders at day 0. Solid symbols denote instances where the number of ASC at that time point is significantly different ($P \leq 0.05$) from the number at the preceding time point (*t* test); this analysis was restricted to values from mice with measurable ASC. ns, not significant.

To further investigate vaccine induced immune responses, mice were intranasally immunized with two doses of FF vaccine at 7-day intervals. At 3 and 7 days after each dose, mononuclear cells were isolated from lungs, Peyer's patches, mesenteric lymph nodes, intestinal lamina propria, and spleen. These were tested for the presence of *Campylobacter*-specific IgA and IgG antibody-secreting cells (ASC); the data are presented in Fig. 4. No ASC were detected before vaccination or at any interval from mice receiving PBS only. Vaccine induced both IgA and IgG ASC responses in all tested tissues and organs, and these responses were ongoing through 7 days after vaccination.

Vaccine efficacy was determined by intranasally challenging mice with *C. jejuni* (81-176) 28 days after primary immunization. Animals were observed for 6 consecutive days, and signs of illness were scored as described above; results are summarized in Table 3. All vaccines provided some protection against *C. jejuni* challenge. The differences among vaccines were not significant ($P > 0.05$). Animals receiving HK or FF formulations were completely protected from intestinal colonization, whereas 17% of the animals immunized with the mixture vaccine remained colonized for more than 6 days.

DISCUSSION

Over the last several years, extensive effort has been directed toward the development of in vivo and ex vivo model systems suitable for the study of *Campylobacter* pathogenesis and immunity. These have included studies of primates (7, 17, 33), dogs (31), pigs (5), calves (4), chickens (16), ferrets (9, 18), guinea pigs (37), hamsters (2, 22), and mice (6, 10, 12, 32). The large number of models speaks to the difficulties encountered in identifying a system that is sufficiently robust to meet research requirements. The difficulties encountered included failure to produce diarrhea (6, 10, 12, 18, 32), lack of availability of sufficient numbers of host animals to allow vigorous investigations (7, 17, 33), lack of reagents or methods for detailed analytic studies (9, 18), relatively rapid changes in resistance to *Campylobacter* with age making the model unstable for the resolution of resistance mechanisms (7, 14, 17, 33), and requirements for special manipulations, including surgical procedures (14, 15) or treatments with chemicals (35), to cause the model to act as wanted. Because the influences of these treatments or surgical procedures on host-parasite interactions, including immune responses, are not well defined, the interpretation of results from studies that employ these approaches are confounded by the unknown impact of these manipulations.

In our laboratories, two animal models have been routinely used to study *Campylobacter* virulence and immunity, the RITARD model (14, 15) and oral challenge of adult mice (6, 32). The mouse oral challenge model was developed, in part, based on previous results of Blaser et al. (12), because of limitations imposed by the rabbit model. In rabbits, diarrhea is commonly seen only in younger animals following infection (14), thus precluding this model's use in long-term vaccine protection studies. In the mouse oral challenge model, no grossly observable disease occurs following challenge, yet intestinal colonization and, at times, dissemination of bacteria to the blood is seen (6). Oral challenge of mice previously exposed to *Campylobacter* antigens or *Campylobacter* infection revealed the existence of an acquired protection to the challenge manifested as reduced or ablated colonization and reduced rates of bacteremia (6).

We initiated studies of the murine intranasal challenge in an attempt to extend the capacities of the oral challenge mouse model to include, in addition to colonization and protection from colonization, grossly observable illness and immune protection against overt illness. We view the capacity to discriminate these two types of protection as critical because studies of humans (11) show that resistance to disease may exist without resistance to colonization. The results reported here demonstrate that this model may provide the first practical means to examine resistance to *Campylobacter* challenge as both protection against illness and protection against colonization. Patterns of bacterial growth and organ distribution as well as signs of illness after intranasal challenge of BALB/c mice with

Campylobacter isolates clearly identify mice with acquired immunity to this enteropathogen. The mechanisms associated with these two manifestations of anti-*Campylobacter* immune protection and their possible relationship to like events in humans remain to be determined. In addition, this model was shown to provide a better and potentially more clinically relevant means to rapidly classify stock reference cultures, as well as recent clinical isolates of this organism based on virulence. Given the exceptional availability of genetically defined mouse strains and their apparent susceptibility to some *C. jejuni* strains, plus the wide range of reagents and methods available for the comprehensive analysis of agent-specific immune responses in mice, this model has potential for use in studies to define the genetic and molecular basis of *Campylobacter* pathogenesis and immunity.

The promise of the murine intranasal challenge system as a suitable model for predicting and analyzing human disease-relevant mechanisms is further supported by several recent studies by others, who have used a similar approach to study *Shigella* virulence and immunity. In the *Shigella* system, the intranasal challenge model has proven useful in evaluating efficacy of candidate vaccines and also a relationship has been found between the extent of pathology and illness following intranasal challenge and that which occurred when the same strains were administered to volunteers (27, 28).

Review of the course of infection and of the evolution of the immune responses resulting from intranasal infection showed that *Campylobacter* rapidly disseminate enterally and parenterally; the *Campylobacter* burden in some tissues was maximal soon after challenge (4 h, the earliest time point tested) and then declined afterwards to apparent clearance. Since *Campylobacter* organisms are not normally found in laboratory-reared BALB/c mice, it is unlikely that the clearance of the bacteria from some tissues within hours to 2 days of challenge is the result of an acquired immunologic resistance to the agent. If this clearance is not the result of an immune response, then it will be necessary for subsequent studies to detect whether this clearance is a nonspecific resistance mechanism that kills the bacteria or a reflection of a preferential out migration of *Campylobacter* organisms from these sites to other tissues or a failure of campylobacters to thrive in these sites.

The establishment and persistence of intestinal colonization after intranasal challenge are similar to the pattern of colonization seen after rectal (9) or oral (6, 10, 12) infection with this bacterium. An additional pattern of infections that are similar between the intranasal model and other models that have used adult mice (12, 35) is the rapid, probably within minutes, dissemination of campylobacters from an enteral site of challenge to parenteral sites as demonstrated by isolation of the agent from blood. In spite of their rapid entry into the blood, bacteria in blood, but not in all deep tissues, fell to below detectable levels within a few days. Whether this rapid systemic dissemination and clearance from blood are common characteristics with human infections is not known and probably would not be detected with the techniques commonly applied in clinics. The work of Skirrow and others (34) suggests that *Campylobacter* bacteremia in humans is substantially underreported, since blood cultures are rarely taken when individuals seek care for diarrhea episodes.

This study found BALB/c mice and *C. jejuni* 81-176 as the preferred host-parasite combination for studies of pathogenesis and immunity. The results of testing this host-parasite pair may be interpreted with caution because the *C. jejuni* 81-176 strain used had, as the result of previous studies in this laboratory (6), been passaged in BALB/c mice before its use in the present study; this was not true for other campylobacters. Further, again because of other previous and ongoing studies (6, 7), the antigens used for immunologic assays and vaccines were prepared from *C. jejuni* 81-176.

Enteropathogenic campylobacters have become increasingly recognized as a significant bacterial cause of human disease (13) and under some conditions are the leading bacterial cause of diarrhea in humans (36, 38). Efforts are under way to reduce the morbidity caused by these pathogens, including attempts at vaccine development (6, 7, 32). Progress toward this goal has been slowed by deficiencies in knowledge of virulence factors and mechanisms of immunity. In ongoing investigations the model presented in this report is being used to direct and monitor the development of candidate *Campylobacter* vaccines, including live attenuated and inactivated whole-cell formulations.

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