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Immune Response of Athymic and Euthymic Germfree Mice to *Campylobacter* spp.

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Pure cultures of several *Campylobacter* spp. induced a specific humoral immune response after they colonized and infected gnotobiotic mice; however, *Campylobacter*-immune mouse serum was not bactericidal (in vitro), manifested a weak agglutination reaction (in vitro), and showed specificity (strain 45100-immune mouse sera) for the homologous (infecting) *Campylobacter* strain, but was not able to passively protect germfree athymic (*nu/nu*) BALB/c mice against *Campylobacter* infection and diarrhea. Active immunization of germfree *nu/nu* mice with Formalin-killed *C. jejuni* also did not protect the gnotobiotic mice from *Campylobacter* infection and diarrhea. It appears from the results of our initial gnotobiotic studies that antibodies in serum against the infecting strain of *C. jejuni* may not play an essential role in resistance to *Campylobacter* disease in mice.

Worldwide, campylobacteriosis is becoming the most common type of enteritis affecting humans. The species most often associated with human illness is *Campylobacter jejuni*. Recent discoveries of new *Campylobacter* species (22, 29) and their possible association with human disease (37, 39) emphasize the need for a suitable animal model to study host-*Campylobacter* interactions so that basic mechanisms involved in the pathogenesis of and resistance to this important enteric pathogen can be studied.

In humans *C. jejuni* causes a variety of clinical symptoms that range from a carrier state to a severe ulcerative colitis (11, 17, 27). The most common form of *Campylobacter*-induced disease in adults is acute colitis (9, 18, 27, 28). The high incidence of *C. jejuni* infection in children less than 2 years of age suggests that the ease of intestinal colonization, immature immune responses, or both may be important factors in susceptibility to the disease. *C. jejuni* has been established as an intestinal pathogen in humans by culture data and by the formation of *Campylobacter*-specific serum antibodies (immunoglobulin G [IgG] and IgM) during the course of an infection (4, 11, 12). Rhesus monkeys (19), gnotobiotic beagle puppies (32), calves (1), rabbits (14), and mice (7) experimentally infected with *Campylobacter* spp. have demonstrated various degrees of humoral responses after challenge.

The relative importance of humoral and cell-mediated immunity in resistance to campylobacteriosis has not yet been delineated. We have developed a gnotobiotic mouse model to study colonization, infection, and disease by *Campylobacter* spp. (43, 44). The purposes of this study were to elucidate the humoral response to *Campylobacter* spp. in orally infected, congenitally athymic and euthymic BALB/c mice and to assess whether antibody plays a role in resistance to campylobacteriosis. We also report on the results of various in vitro assays on the capacity of *Campylobacter*-immune serum (mouse and rabbit) to kill, inhibit motility, and bind to *Campylobacter* proteins.

MATERIALS AND METHODS

Mice. Eight-week-old BALB/c athymic (*nu/nu*) and euthymic (+/*nu*) germfree (GF) mice were used in these studies. They were reared, housed, and colonized with a pure culture of *Campylobacter* in plastic isolators at the Gnotobiotic Research Laboratory, University of Wisconsin, Madison.

Adult defined-flora (DF) *nu/nu* and +/*nu* BALB/c mice were also used in the immunization experiments. These mice were colonized with several known species of gram-positive bacilli and cocci and were immunized to produce *Campylobacter*-immune mouse serum.

Bacteria. *C. jejuni* 45100 was used in these studies. It was originally isolated from the feces of a 2-month-old child (female) with diarrhea, and was obtained from the Wisconsin State Laboratory of Hygiene, Madison. This strain was intestinally adapted by its continuous presence in the intestinal tract of gnotobiotic BALB/c mice for 2 years and has been shown to cause diarrhea in *nu/nu* BALB/c mice (44). Strain 45100 was characterized (44) as a weak cytotoxin-producing strain when tested in a modification of a CHO cell assay described by Guerrant et al. (23).

Other *Campylobacter* spp. that were used to colonize GF BALB/c mice were *C. jejuni* 24, *C. jejuni* INN-73-83, and *C. fetus* subsp. *fetus* 255. Strains 24 and 255, also obtained from the Wisconsin State Laboratory of Hygiene, were isolated from human feces and blood, respectively. Strain INN-73-83 was obtained from Frederick Klipstein, University of Rochester Medical Center, Rochester, N.Y., and was included in this study as a documented heat-labile enterotoxin-producing strain of *C. jejuni* which was originally isolated from a Mexican child with diarrhea (25). The maintenance and growth of these *Campylobacter* strains have been reported previously (43, 44).

Quantitation of bacteria in tissues. At various times after oral inoculation, three *nu/nu* mice, three +/*nu* mice, or both were anesthetized (ether) and exsanguinated by cardiac puncture, and the internal organs were aseptically removed. Organs cultured for viable *C. jejuni* 45100 included mesenteric lymph nodes (MLNs) from individual mice and pooled ceca from either three *nu/nu* mice monoassociated (MA) with a *Campylobacter* strain or three +/*nu* mice MA with a *Campylobacter* strain at each time interval. Tissues were homogenized in 5 ml of phosphate-buffered saline (PBS; pH 7.2) and serially diluted in PBS (10-fold), and duplicate 0.05-ml amounts were plated onto 5% sheep blood agar plates (SBAPs). Separate 1.0-ml fractions from each MLN

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homogenate were added to 9 ml of brucella broth (Difco Laboratories, Detroit, Mich.) for enrichment and to a preweighed aluminum pan for subsequent drying and weighing. All plating media and enrichment broths were incubated at 42°C for 48 h in a microaerophilic atmosphere produced inside vented jars by evacuation (25 lb/in^2) and replacement (80% N_2, 10% CO_2, and 10% H_2). Enrichment broths were then subcultured onto SBAPs and incubated as described above to determine the presence of viable *Campylobacter* spp.

Serological techniques. (i) Serum. Serum was obtained from *Campylobacter*-MA mice at various times after oral inoculation. The serum from each of three mice (*nu/nu* or +/*nu*) at each sampling period was pooled and stored at −70°C before testing. The indirect fluorescent antibody (IFA) assay used to detect anti-*Campylobacter* antibodies in serum, saliva, or gut washings was similar to the one used by Blaser and co-workers (4). The *Campylobacter* antigen (whole cell) used in the IFA assay was prepared by the method of Garvey et al. (21). A 1:100 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, IgM, or IgA (Meloy Laboratories, Inc, Springfield, Va.) or FITC-labeled goat anti-rabbit IgG (Dynatech Diagnostics, South Windham, Mass.) served as the conjugate in this assay. Fluorescence readings were made with a vertical fluorescence microscope (AO; Spencer) fitted with a 100-W mercury lamp and a filter (AO 2072; Spencer) on each dilution. A minimum of 1+ fluorescence in a particular dilution was read as positive.

(ii) Saliva. Saliva was collected from *nu/nu* or +/*nu* mice that were long-term MA (~10 months) with *C. jejuni* 45100 or *C. fetus* subsp. *fetus* 255 by sedating the mice with an injection (intraperitoneally [i.p.]) of Nembutal (0.06 mg/g; Abbott Laboratories, North Chicago, Ill.) and then by immediately injecting (subcutaneously) approximately 0.2 ml of urecholine (1:200 dilution of a 5-mg/ml stock). The saliva was collected in a plastic test tube, pooled, diluted (1:4, 1:5, or 1:10) in PBS, and frozen at −70°C until tested for anti-*C. jejuni* antibodies (IgG, IgM, or IgA) by the IFA assay described above.

(iii) Gut washings. Intestinal washings were collected from *nu/nu* and +/*nu* BALB/c mice that were long-term MA (~10 months) with *C. jejuni* 45100 and *C. fetus* subsp. *fetus* 255. The small intestine (duodenum to distal ileum) and colons were aseptically removed and placed into separate sterile petri dishes, and 2 ml of PBS was gently forced through each intestinal segment. After all of the contents and liquid were massaged out into the dish, the washings were centrifuged at 12,000 × *g* at 4°C for 30 min, and the supernatants were frozen at −70°C until tested by the IFA assay for IgG, IgM, or IgA against *C. jejuni*.

Colon tissue sections. Direct FITC staining of serial sections from Epon-embedded colons taken from gnotobiotic *nu/nu* and +/*nu* BALB/c mice that were colonized with *Campylobacter* spp for 8 days were processed as described previously (44).

Spleen and cecal size. At various times after colonization with *Campylobacter* spp. mice were anesthetized with ether. Individual spleens and ceca were then aseptically removed and weighed. The relationship of spleen or cecal weight to total body weight was expressed by use of the following formula: (organ weight)/(total body weight) × 100.

Mouse immunizations. (i) Inoculum preparation and immunization schedule. The inoculum of *C. jejuni* that was used to immunize *nu/nu* GF BALB/c mice and to produce immune serum from *nu/nu* and +/*nu* DF BALB/c mice (for use in passive protection experiments) was prepared as described by Garvey et al. (21). The immunization schedule was as follows. At time zero each mouse was injected (i.p.) with 0.5 ml of the 0.3% formalinized *Campylobacter* spp. Thereafter, on days 5, 11, and 18 the mice received booster injections (i.p.) of 0.25 ml at each time interval. GF and DF BALB/c mice receiving injections of 0.3% Formalin-PBS served as controls. Serum was collected from the various groups of immunized mice (GF and DF) 18 and 25 days after the initial immunization and assayed for specific IgG to *C. jejuni* 45100 by the IFA assay.

(ii) Active immunization. Twenty-eight days after the first injection (i.p.), six GF *nu/nu* mice actively immunized with 45100 and six PBS-treated controls were transferred into an isolator which housed *C. jejuni* 45100 MA mice. The immunized and control mice were rapidly (<1 day) colonized with viable *C. jejuni* 45100 through a natural fecal-oral route. Three immunized 45100 and three control (PBS-treated) mice were killed on days 7 and 35 after colonization with *Campylobacter* spp., and the following information was recorded: cecal weights, spleen weights, MLN viable counts, pooled cecal viable counts, and IgG titers in serum to *C. jejuni* 45100 by the IFA assay.

(iii) Passive immunization. Immune sera from *Campylobacter*-immunized BALB/c mice or rabbits (44) were used in experiments designed to assess the effect of antisera on campylobacteriosis in *nu/nu* mice. Passive protection was carried out as follows. Six groups of three 8- to 10-week-old GF *nu/nu* BALB/c mice were each injected (i.p.) with 0.5 ml of one of the following: (i) PBS; (ii) normal rabbit serum (NRS; undiluted); (iii) hyperimmune strain 45100 rabbit serum (HRS; to strain 45100, undiluted); (iv) serum from DF +/*nu* mice treated with PBS (diluted 1:5 in PBS); (v) hyperimmune mouse serum from DF +/*nu* mice injected with strain 45100 (diluted 1:5 in PBS); (vi) strain 45100-MA (10 month) +/*nu* mouse serum (diluted 1:5 in PBS). Immunization groups i, ii, and iv served as controls. After the injections, all mice were immediately inoculated orally with *Campylobacter* spp. with a swab containing a fresh fecal suspension (10^8 to 10^9 CFU/ml) from BALB/c mice that were MA with *C. jejuni* 45100. Three days after the initial i.p. injection of antisera and *Campylobacter* monoassociation, a booster immunization (0.25 ml i.p.) of the appropriate antiserum (or PBS) was given to each mouse. All of these immunizations were carried out by aseptic techniques in a laminar flow hood (SterileGard; The Baker Co., Sanford, Maine). The athymic mice were housed inside the hood in groups of three in sterile cages fitted with sterile bonnets. The mice were fed autoclaved commercial mouse chow (5010C; Ralston Purina Co., St. Louis, Mo.) and water ad libitum. A UV light in the laminar flow unit remained on during the experiment to decrease the risk of airborne microbial contamination. After colonization with *C. jejuni* 45100 for 7 days, all mice were killed and the following information was recorded: cecal weights, spleen weights, viable bacteria present in individual MLNs and pooled cecal contents, and antibody (IgG) titers to *C. jejuni* 45100 in serum. Quantitative cultures of bacteria in tissues and in the alimentary tracts were done as described above with SBAPs (GIBCO Diagnostics, Madison, Wis.).

Specificity of sera from *Campylobacter*-colonized mice. Serum samples from both *nu/nu* and +/*nu* mice that were MA for 36 days with *C. jejuni* 45100 were tested for specific binding to the homologous strain 45100, heterologous *C. jejuni* 24 and INN-73-83, or *C. fetus* subsp. *fetus* 255 by the IFA assay described above.

Serum bactericidal assay. The serum bactericidal assay used in this study was a microtiter adaptation of an assay described by Karmali and Fleming (24). The serum samples tested were from *nu/nu* and +/*nu* mice that were MA for 36 or 224 days with *C. jejuni* or from rabbits immunized with HRS strain 45100. Freshly diluted (1:10 in chilled PBS) guinea pig complement (Cordis Laboratories, Miami, Fla.) checked daily for activity in a sheep erythrocyte–anti-sheep erythrocyte system was used in the assay. The *Campylobacter* inoculum consisted of ~5 × 10^3 CFUs per microtiter well of either *C. jejuni* 45100 or *C. fetus* subsp. *fetus*. After incubation at 37°C for 1 h in a moist CO_2 (5%) atmosphere, the number of CFUs from wells with anti-*Campylobacter* antibodies and complement were compared with controls that consisted of PBS, PBS-complement, GF *nu/nu* serum, GF +/*nu* serum, and NRS.

Motility inhibition with immune mouse serum. The effect of immune mouse serum or HRS on *Campylobacter* motility was determined in an in vitro assay. Immune sera, collected from *nu/nu* and +/*nu* BALB/c mice that were MA for 36 days with *C. jejuni* 45100 and HRS to *C. jejuni* 45100 were tested for their ability to inhibit motility of the homologous strain of *C. jejuni* 45100 in vitro. Serum from GF *nu/nu* and GF +/*nu* mice and NRS were included as negative controls. The *C. jejuni* 45100 inoculum was prepared by adjusting, in sterile PBS, the overnight surface growth of a pure culture of the organism from a SBAP to a MacFarland no. 4 turbidity standard. Equal amounts (0.05 ml) of each test and control serum sample were mixed with the viable *Campylobacter* suspension on a clean glass microscope slide, a cover slip was added, and the organisms were immediately observed (under a light microscope) for inhibition of motility in test and control (nonimmune serum) mice over a period of 15 min.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Crude whole cell protein extracts of *C. jejuni* were studied by the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) methods of Laemmli (26) with adaptations by Nachamkin and Hart (30). The bacterial culture extracted with SDS was a +/*nu* mouse-adapted strain of *C. jejuni* 45100 that was grown on 5% SBAPs at 42°C in a microaerophilic atmosphere for 48 h. The surface growth was harvested and suspended in Tris buffer (pH 7.7), and the turbidity was adjusted to an optical density of 2.0 at 540 nm. A second SDS-treated *C. jejuni* 45100 antigen consisted of an old (1 year) formalinized (0.3% Formalin-PBS) suspension of organisms which was processed by the same procedures as described above for fresh cells. The two bacterial suspensions were diluted 1:1 with SDS-PAGE-solubilizing buffer and boiled for 5 min, and 40-μl amounts were applied to the gel. The crude whole-cell proteins were electrophoresed on a 4% stacking gel and a 12% running gel. Protein electrophoresis was performed with a slab gel apparatus (Bio-Rad Laboratories, Richmond, Calif.) at 40 mA, until the tracking dye entered the resolving gel, and at 400 V through the running gel. Proteins were visualized with Coomassie blue stain. Known proteins were run on each gel to estimate the molecular weight of the *Campylobacter* proteins extracted for the immunoblotting experiments.

Immunoblotting. After SDS-PAGE the separated proteins were immediately transferred from slab gels to nitrocellulose paper by the method of Towbin et al. (40). The blot transfers were carried out with a transblot apparatus (Bio-Rad) in 25 mM Tris buffer (pH 8.3) overnight at 30 V in a refrigerator (4°C). A comparison of amido black-stained nitrocellulose blot transfers to Coomassie blue-stained slab gels demonstrated that the *Campylobacter* proteins were efficiently transferred to the nitrocellulose paper. After transfer, the nitrocellulose paper was coated with 3% bovine serum albumin in PBS for 1 h at 37°C to block nonspecific binding sites. The paper was then placed for 2 h into a 1:20 dilution of mouse serum (+/*nu* GF control, *nu/nu* GF control, +/*nu* serum that was MA for 10 months with *C. jejuni* 45100, or *nu/nu* serum that was MA for 10 months with *C. jejuni* 45100), followed by four 10-min rinses in PBS (at room temperature). The nitrocellulose paper was then incubated at room temperature for 1 h with a 1:320 dilution in 3% bovine serum albumin–PBS of a peroxidase-conjugated goat anti-mouse IgG (heavy and light chains; U.S. Biochemical Corporation, Cleveland, Ohio), followed by four 10-min washes in PBS. The substrate, consisting of 4 chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo.), was added to the nitrocellulose papers and rinsed off in approximately 15 min (after color developed).

TABLE 1. Antibody (IgG and IgM) response in serum of BALB/c mice after colonization and infection with a pure culture of *Campylobacter*

BALB/c genotype and wk after oral challenge[a]	Antibody titer in sera of mice MA with[b]:							
	C. jejuni 45100		*C. jejuni* 24		*C. jejuni* INN-73-83		*C. fetus* subsp. *fetus* 255	
	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
nu/nu								
0	—[c]	—						
0.5	—	—	—	—	—	—	—	—
1	—	1:5	—	—	1:5	1:10	—	—
1.5	—	1:5					1:5	—
2			—	1:5	1:10	1:10		
3	1:5	1:5	1:5	—				
5	1:20	1:5			1:20	1:10		
6							1:40	—
32	1:320	1:5					1:160	—
+/*nu*								
0	—	—						
0.5	—	—	—	—	—	—	—	—
1	—	1:5	—	—	1:5	1:10	—	—
1.5	1:5	1:5					—	—
2			—	—	1:10	1:10		
3	1:10	1:5	1:10	1:5				
5	1:40	1:5			1:40	1:10		
6							1:40	1:5
32	1:320	1:5					1:160	—

[a] *nu/nu* = athymic; +/*nu* = euthymic.
[b] Antibody titer was assessed by the IFA assay of pooled sera from three mice against the homologous *Campylobacter* strain at each interval.
[c] —, No fluorescence detected with the lowest serum dilution (1:5).

Data presentation and statistical methods. Viable counts (log_{10} CFU/g) of viable *Campylobacter* spp. and cecal and spleen weights of BALB/c mice are presented as means ± standard error of the means. A statistical analysis of these data was done using a one-way analysis of variance, employing the Fischer least-significant-difference test.

RESULTS

Serology of *Campylobacter*-colonized mice. (i) Serum antibodies. Table 1 shows data on the antibody (IgG and IgM) responses of gnotobiotic mice following intestinal colonization and infection with *Campylobacter* spp. In general, both *nu/nu* and +/*nu* BALB/c mice, when intestinally colonized with a pure culture of *Campylobacter*, responded by produc-

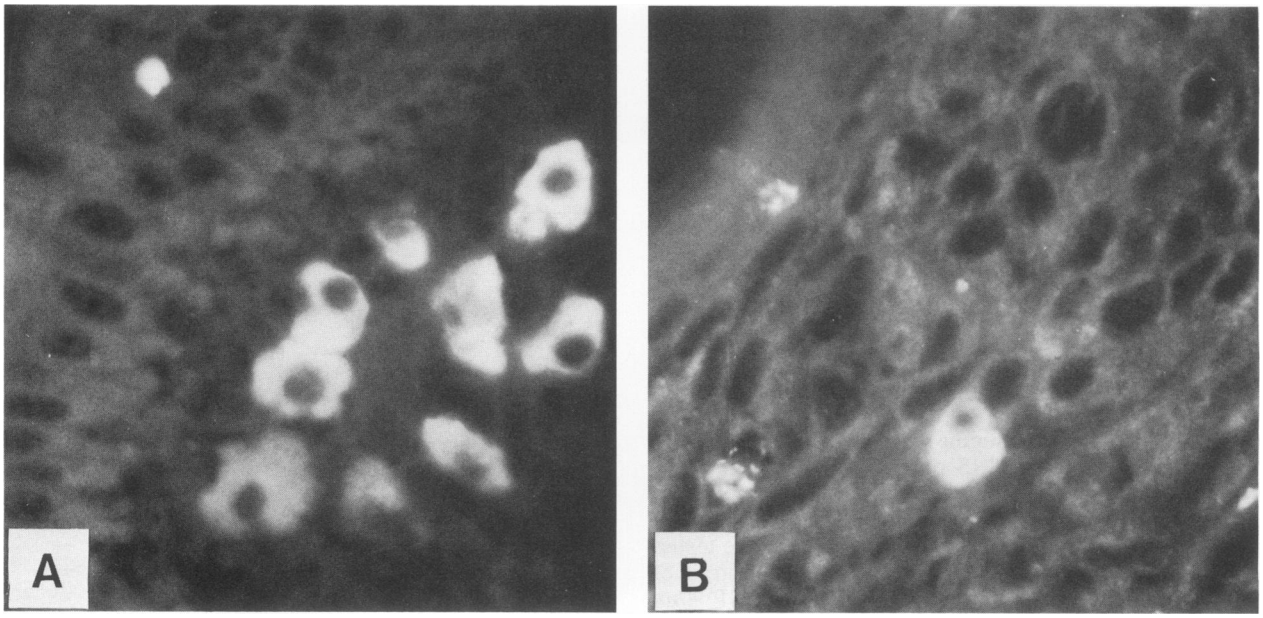

FIG. 1. Direct immunofluorescence of IgA-bearing cells in the colonic lamina propria of euthymic (A) and athymic (B) BALB/c mice which were colonized by *C. jejuni* 45100 for 7 days. The FITC was conjugated to a goat anti-mouse IgA. Magnification, ×1400.

ing IgG in serum (titers of 1:5 to 1:320) and, to a lesser extent, IgM (titers of 1:5 to 1:10) to the homologous colonizing strain. No IgA was detected by the IFA assay in serum samples from *Campylobacter*-MA mice.

(ii) Antibodies in saliva and gut washings. Pooled saliva collected from *nu/nu* and +/*nu* mice that were MA (~10 months) with *C. jejuni* 45100 had no detectable antibodies (IgG, IgM, or IgA) against *C. jejuni* 45100 by the IFA assay. Saliva from +/*nu* mice that were MA with *C. fetus* subsp. *fetus* 255 contained IgA antibodies (1:4 titer) against the homologous (colonizing) strain of *C. fetus* in the IFA assay.

IgG and IgA against *C. jejuni* 45100 were detected (1:4 titers) in colon washings from +/*nu* mice that were colonized with *C. jejuni* 45100 for 10 months; however, only one of three and two of three +/*nu* mice tested were positive for IgG and IgA, respectively, in colonic washings. All small intestinal and colonic washings tested by immunofluorescence were negative for IgM to *C. jejuni* 45100. Saline washings from the small intestines and colon of *nu/nu* and +/*nu* mice colonized with *C. fetus* were positive for IgA (1:4) to *C. fetus* subsp. *fetus* 255.

Direct FITC staining of colonic tissue for immunoglobulins. Athymic GF BALB/c mice consistently developed diarrhea 7 days after intestinal colonization with a mouse-adapted strain of *C. jejuni* 45100, whereas heterozygous mice did not (43, 44). To ascertain whether an immune response occurred in situ at the mucosal (lamina propria) level in the *Campylobacter*-colonized mice at the time of the diarrhea response, we used direct FITC staining to detect immunoglobulin-bearing cells in the colonic lamina propria of colonized mice. Serial sections (3 μm) of colon tissue from *nu/nu* and +/*nu* mice that were MA for 7 days with mouse-adapted *C. jejuni* 45100 were stained with FITC-labeled goat anti-mouse IgG, IgM, or IgA. The +/*nu* mice had many cells (clusters of 6 to 12) in their lamina propria that were positive for mouse IgA (GF controls had a rare IgA-positive cell), while the *nu/nu* mice that were MA for 7 days had only a rare IgA-positive cell(s) in their lamina propria (Fig. 1). The fluorescence of these cells (IgA bearing) was blocked when unconjugated rabbit anti-mouse IgA was used to precoat the tissue secretions before they were stained with the FITC-labeled goat anti-mouse IgA. The numbers of cells with IgG and IgM in the colonic lamina propria of *C. jejuni*-colonized (7 days) *nu/nu* and +/*nu* mice (detected by staining with FITC) were minimal (they were present in occasional cells scattered throughout the lamina propria). We were not able, using the FITC-labeling method, to detect mouse IgG, IgM, or IgA coating the *Campylobacter* organisms visible in the colon tissue sections from either *nu/nu* and +/*nu* that were MA with *C. jejuni*.

Immunoblotting. An SDS extract of *C. jejuni* 45100 whole cells had many different (>40) protein bands on the slab gel stained with Coomassie blue (Fig. 2). The SDS extracts of fresh viable cells (Fig. 2, lane 1) had more protein bands than did the SDS extracts of formalinized *C. jejuni* (Fig. 2, lane 2). Heavy protein bands appeared to be localized in three major areas on the gel that corresponded to molecular sizes of ~60 and ~46 kilodaltons (kDa) and a small peptide fraction at the bottom of the gel (Fig. 2).

GF control mouse sera (IgG) from both +/*nu* (Fig. 3, lane 2) and *nu/nu* (Fig. 3, lane 8) mice reacted weakly with three protein bands (molecular sizes of ~40, ~50, and ~75 kDa) from the SDS extract of a suspension of viable (48 h) *C. jejuni* 45100; serum samples from *nu/nu* and +/*nu* mice that were MA with *C. jejuni* 45100 also had IgG in serum that reacted with the same three bands. It appeared that IgG in sera from +/*nu* mice that were MA for 10 months with *C. jejuni* 45100 (Fig. 3, lane 4) reacted more intensely with ~15 SDS-extracted (from fresh viable cells) protein bands than did sera from MA (10 months) *nu/nu* (Fig. 3, lane 6) mice that reacted with ~8 bands; however, sera from MA *nu/nu* and +/*nu* mice (Fig. 3, lanes 4 and 6, respectively) had antibody that reacted with several bands (at ~100, ~60, ~45, and ~29 kDa and a small peptide fraction at the bottom of the gel). GF control sera (Fig. 3, lanes 2 and 8) did not react with the latter protein bands of *C. jejuni*.

The SDS-extracted proteins prepared from a formalinized suspension of *C jejuni* (Fig. 3, lanes 1, 3, 5, and 7) did not react with sera from GF +/*nu* or *nu/nu* mice (Fig. 3, lanes 1 and 7, respectively) and showed fewer bands when exposed to sera from *nu/nu* and +/*nu* mice that were MA with *C. jejuni* (Fig. 3, lanes 3 and 5, respectively) than SDS extracts of fresh cells (Fig. 3, lanes 4 and 6).

Bactericidal assay and motility inhibition with immune sera. HRS to strain 45100 had a *C. jejuni* 45100 bactericidal titer of 1:32; however, all GF (control) and *Campylobacter*-immune mouse sera tested were unable to kill *C. jejuni* 45100 or *C. fetus* subsp. *fetus* 255 in our bactericidal assay. Nonimmune rabbit and GF mouse sera (*nu/nu* and +/*nu*) had no noticeable effects on the motility of *C. jejuni*; however, the immune rabbit sera (strain 45100) caused an immediate (<1 min) agglutination of the motile *C. jejuni* 45100, which progressed with time (clumps increased in size). The two immune (strain 45100) mouse serum samples tested caused a slight (weak) motility inhibition of *C. jejuni* 45100 by the end of the 15-min observation period.

Specificity of mouse antiserum for *Campylobacter* spp. When *nu/nu* mouse sera immune to *C. jejuni* 45100 were tested against whole-cell antigen preparations from *C. jejuni* 24, *C. jejuni* INN-73-83, and *C. fetus* subsp. *fetus* 255, no cross-reactions were noted in the IFA assay that we used. Sera from *C. jejuni* 45100-colonized +/*nu* mice did cross-react (1:5 titer) slightly with *C. jejuni* INN-73-83 cells. The sera from strain 45100-colonized *nu/nu* mice did not cross-react with the heterologous *C. jejuni* INN-73-83 antigen.

Passive and active immunization. The antibody response of GF or DF BALB/c mice to i.p. immunization with formalinized *Campylobacter* is shown in Table 2. Passive immunizations of *nu/nu* BALB/c mice with either HRS (IgG titer by the IFA assay, ≥1:2,056) or hyperimmune +/*nu* mouse serum (Ig titer by the IFA assay, 1:256) did not prevent *C. jejuni* 45100 from causing diarrhea, infecting the MLNs (~5.5 $\log_{10}$ CFU/g), or reducing the size of the ceca (Table 3). *Campylobacter*-induced disease in these mice occurred in spite of the fact that at 7 days after oral challenge these nude mice had detectable titers in serum for either rabbit IgG (1:8) or mouse IgG (1:2) to *C. jejuni* 45100. The only sign that the passive administration of antibody protected *nu/nu* mice from campylobacteriosis was that those treated with hyperimmune mouse sera showed no significant splenomegaly following oral challenge (Table 3).

In another experiment, GF *nu/nu* BALB/c mice were actively immunized with a Formalin-killed suspension of *C. jejuni* 45100 and then were orally challenged (intestinally MA) with *C. jejuni* 45100. The actively immunized mice were not protected from colonization, infection, or disease (Table 4). Seven days after oral challenge, actively immunized (with formalinized *C. jejuni* 45100 and nonimmunized control *nu/nu* mice had approximately the same number of viable *C. jejuni* 45100 isolates in their MLNs, experienced a cecal shrinkage, and had significant splenomegaly. At 35 days after oral challenge with strain 45100, the actively immunized *nu/nu* mice and the nonimmunized control mice had similar numbers of viable *C. jejuni* 45100 in their MLNs and a normal cecal and spleen size. Diarrhea was evident in mice actively immunized with strain 45100, in spite of the fact that these adult *nu/nu* BALB/c mice had detectable IgG titers in serum to *C. jejuni* 45100 prior to oral challenge (1:2) and at day 7 (1:2) after oral challenge, as determined by the IFA assay. Conversely, the control (PBS-treated) mice had no detectable anti-*Campylobacter* IgG in serum at time zero or at day 7 after oral challenge. By day 35 after oral challenge the titer increased to 1:8 in control (PBS-treated) mice and

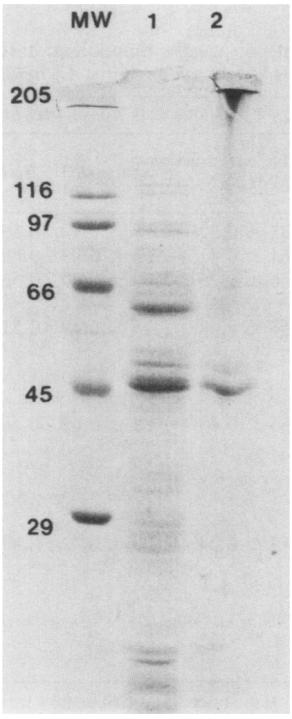

FIG. 2. Coomassie blue stain of SDS-extracted proteins prepared from either a fresh (48 h) (lane 1) or a formalinized (lane 2) suspension of *C. jejuni* 45100. Molecular weight (MW) markers are indicated to the left, in thousands.

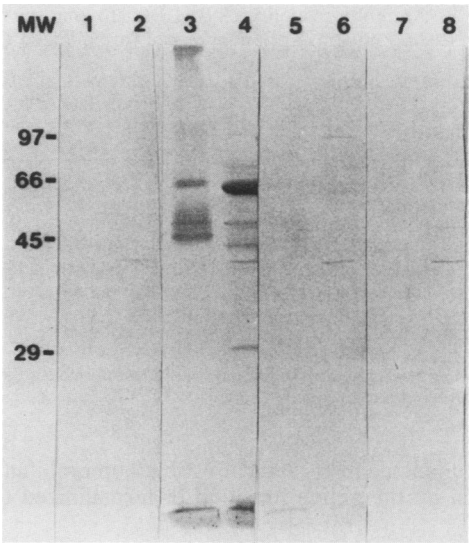

FIG. 3. Western blot of mouse IgG in serum against *C. jejuni* 45100 proteins prepared from SDS extracts of either freshly grown cells (lanes 2, 4, 6, and 8) or formalinized cells (lanes 1, 3, 5, and 7). The BALB/c mouse sera (1:20) were added as follows: GF +/*nu*, lanes 1 and 2; +/*nu* mice MA with *C. jejuni* 45100 for 10 months, lanes 3 and 4; *nu/nu* mice MA with *C. jejuni* 45100 for 10 months, lanes 5 and 6; GF *nu/nu* mice, lanes 7 and 8. The second antiserum (added to all lanes) was a 1:320 dilution of goat anti-mouse IgG (peroxidase conjugated). Molecular weights (MW) are indicated to the left, in thousands.

TABLE 2. Antibody response of BALB/c athymic (*nu/nu*) and euthymic (+/*nu*) mice to i.p. injections of formalinized *C. jejuni* 45100[a]

Microbial status (BALB/c genotype)	Antibody (IgG) titer on day:	
	18	25
GF (*nu/nu*)	1:2[b]	ND[c]
DF (*nu/nu*)	1:4	1:32
DF (+/*nu*)	1:64	1:256

[a] Groups of six *nu/nu* and six +/*nu* BALB/c mice received initial injections (i.p.) of 0.5 ml of formalinized *C. jejuni* 45100 or PBS at time zero; thereafter, on days 5, 11, and 18 all mice received booster injections (i.p.) of 0.25 ml of the appropriate inoculum. GF and DF mice and control mice injected with PBS were negative for anti-*Campylobacter* IgG.

[b] Titers were determined by IFA assay with goat anti-mouse IgG.

[c] ND, Not determined.

1:32 in immunized mice, indicating that humoral immunity is stimulated by the active injection of formalinized *C. jejuni* 45100.

DISCUSSION

To date, little is known about the relative importance of cellular and humoral mechanisms in resistance to *Campylobacter* spp. Recently, Blaser and colleagues (5) demonstrated that clinical campylobacteriosis induces a rise in the antibody titer to *C. jejuni* in serum. Results of previous studies (2, 3, 15, 16, 31) have also shown that GF mice and rats undergo a normal, but slower, humoral and cell-mediated immune response after oral challenge with various antigens. Results of our serological studies of gnotobiotic *nu/nu* and +/*nu* BALB/c mice that were MA with *Campylobacter* spp. indicated that a humoral response (IgM and IgG) occurred in both mouse genotypes following alimentary tract colonization and infection. We have previously reported (43, 44) that diarrhea occurred in gnotobiotic athymic mice ~7 days after oral challenge with a mouse-adapted strain of *C. jejuni* 45100. In this study we detected a delay in the IgG immune response in serum of *nu/nu* mice to *Campylobacter* spp. (3 weeks after challenge) when compared with their euthymic littermates (1.5 weeks after challenge), which showed no signs of campylobacteriosis. The *nu/nu* mice recovered from disease and eventually produced *Campylobacter*-specific IgG in serum equal (in titer) to that of their +/*nu* counterparts. In our experiments, both *nu/nu* and +/*nu* BALB/c mice developed comparable IgG titers in serum, as determined by the IFA assay, to the colonizing strain of *C. jejuni*; however, Western blot analysis showed that +/*nu* mouse sera reacted to a greater degree and apparently with a larger number of the SDS-extraced *C. jejuni* proteins than did sera from *nu/nu* mice.

We could not show in vitro bactericidal activity with sera from MA *nu/nu* or +/*nu* BALB/c mice against the homologous (i.e., colonizing) strain of *C. jejuni*. Blaser et al. (6) have also reported that neither normal nor immune HA-ICR mouse serum had bactericidal activity against *C. jejuni* and concluded that the bactericidal activity has little or no role in murine resistance to *Campylobacter* bacteremia. Immunity to campylobacteriosis in our gnotobiotic mouse model does not appear to be dependent on bactericidal activity (in vitro) in serum against *Campylobacter* spp. However, results of recent reports indicate that the bactericidal activity in human serum may limit systemic invasion by *C. jejuni* in the disease in humans (10, 11).

Bacterial motility has been shown to be an essential factor for cecal colonization by *Roseburia cecicola* (36) and adherence to ileal epithelial brush borders by *Vibrio cholera* (B. W. Petschow and M. N. Guentzel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B104, p. 118). Antiflagellar antibodies produced by a host may be important in blocking mucous penetration, adherence, and therefore, the colonization of the intestinal tract. Results of a recent study by Caldwell and co-workers (13) also suggest that flagella may play a role in campylobacter virulence. We have demonstrated that antiserum from both *nu/nu* and +/*nu* BALB/c mice that were MA with *Campylobacter* species agglutinated the homologous *C. jejuni* strain in vitro. The importance of these agglutinating antibodies in serum in vivo is not known, but results of recent studies (30, 41) utilizing Western blot analysis of convalescent human sera collected after *C. jejuni* enteritis have suggested that the *Campylobacter* flagellar protein (62 to 66 kDa) is the dominant antigen recognized during infection. We were also able to detect immune responses in antisera of both *nu/nu* and +/*nu* BALB/c mice that were MA with *Campylobacter* spp. that were specific not only for a band of protein that corresponds to the flagellar protein of *C. jejuni* (38, 41) but also for a small protein fraction at the bottom of the gel.

Even though we observed that mouse antiserum could agglutinate *Campylobacter* spp. and specifically bind to several protein components extracted from whole cells, we were not able to passively protect *nu/nu* GF mice from *Campylobacter* disease with injections (i.p.) of specific antisera (mouse or rabbit). Active immunization (i.p.) of GF *nu/nu* BALB/c mice with a Formalin-killed suspension of *C. jejuni* also did not protect the mice from *Campylobacter* infection or disease. We also noted that GF *nu/nu* BALB/c mice responded slower to injections (i.p.) of Formalin-killed *C. jejuni* than did DF *nu/nu* BALB/c mice. One possible explanation for the failure to actively protect the *nu/nu* mice from campylobacteriosis may be that the formalinized *Cam-*

TABLE 3. Inability of passive immunization to protect athymic (*nu/nu*) BALB/c mice against *C. jejuni* 45100

Antisera used for passive immunization	Pathogenesis 7 days after oral challenge			
	Viable bacteria in MLNs[a]	Cecal size[b]	Spleen size[c]	No. with diarrhea/ no. tested
PBS	5.33 ± 0.32	1.78 ± 0.12	0.59 ± 0.04	3/3
NRS	5.64 ± 0.41	1.65 ± 0.03	0.60 ± 0.08	3/3
HRS to strain 45100	5.26 ± 0.26	1.69 ± 0.09	0.56 ± 0.05	3/3
Normal mouse (+/*nu*; PBS treated)	5.54 ± 0.43	2.15 ± 0.49	0.51 ± 0.02	3/3
Hyperimmune mouse (+/*nu*; to strain 45100)	5.66 ± 0.45	2.87 ± 0.62	0.58 ± 0.06	3/3
Hyperimmune mouse (+/*nu*; colonized strain 45100)	5.52 ± 0.24	1.99 ± 0.42	0.47 ± 0.01	3/3

[a] Mean of results for three mice; $\log_{10}$ CFU/g (dry weight) ± standard error of the mean (SEM) of viable *C. jejuni* in the MLNs.

[b] (Cecal weight [g]/total body weight [g]) × 100; mean of results for three mice ± SEM.

[c] (Spleen weight [g] Total body [g]) × 100; mean of results for three mice ± SEM.

TABLE 4. Inability of active immunization with formalinized *C. jejuni* 45100 to protect athymic BALB/c mice against *Campylobacter* pathogenesis

Days after oral challenge	Pathogenesis					
	Viable bacteria in MLN[a]		Cecal size[b]		Spleen size[c]	
	Control (PBS treatment)	Immune to 45100	Control (PBS treatment)	Immune to 45100	Control (PBS treatment)	Immune to 45100
7	5.42 ± 0.13	4.62 ± 0.47	2.05 ± 0.71	1.71 ± 0.02	0.59 ± 0.08	0.60 ± 0.07
35	4.09 ± 0.20	5.75 ± 0.38	5.23 ± 0.68	5.43 ± 0.86	0.33 ± 0.05	0.46 ± 0.01

[a] Log_{10} CFU/g dry weight ± SEM of *C. jejuni* in MLNs; mean ± SEM for three mice at each time interval.
[b] (Cecal weight [g] total body weight [g]) × 100 mean of results for three mice at each time interval ± SEM.
[c] (Spleen weight [g] total body weight [g]) × 100; mean of results for three mice at each time interval ± SEM.

pylobacter inoculum was altered antigenically or did not contain the essential antigens needed for protection against *Campylobacter* infection and disease. Results of our western blot analysis of antibody to Formalin-killed *C. jejuni* SDS-extracted proteins showed no major protein band corresponding to the flagellar protein (~66 kDa) (30) reacting with the +/*nu* mouse antiserum; however, this could have been due to a Formalin-induced cross-linking of the proteins and their failure to enter the gel. Recently, Price and co-workers (33) were successful in using rabbit antisera to heat-treated (100°C) but not to Formalin-treated, *Campylobacter* spp. to detect *C. jejuni* in human stool and rectal biopsy specimens. This is further evidence that Formalin treatment of *C. jejuni* alters its antigenicity. Therefore, in our study, mice actively immunized with a Formalin-killed antigen preparation may have been responding to antigenic components that were not critical for protection.

The antibodies binding to the various strains of *Campylobacter* spp. in the IFA assay appear recognize specific surface components on the various *Campylobacter* spp. because very few cross-reactions were noted in our specificity assays. Butzler and Skirrow (12), using a tube agglutination assay, showed that only 2 of 20 human *C. jejuni* isolates cross-reacted, indicating the existence of many serotypes. Therefore, reinfection by a different *Campylobacter* strain is a likely possibility. It appears that humans infected with *C. jejuni* respond immunologically to various bacterial constituents such as lipopolysaccharide outer membrane proteins and flagellin (8, 30, 41). In this study, a *Campylobacter*-specific IgG response also occurred in serum in *C. jejuni* colonized and infected +/*nu* and *nu*/*nu* BALB/c mice.

Secretory IgA is a key factor in a protective host immune response at the mucosal surface (20, 38, 42). We were not able to detect anti-*Campylobacter* IgA in the serum of colonized and infected gnotobiotic mice. The possibility still exists that secretory IgA at the epithelial cell level in the gut mucosa is a major defense mechanism against campylobacteriosis. We were able to show specific IgA in colon washings from +/*nu* mice that were MA with *C. jejuni* 45100 and *C. fetus* subsp. *fetus* 255 for 10 months but not from *nu*/*nu* mice colon washings. Congenitally athymic mice lack a functional T-cell immunity and therefore are unable to produce specific IgA and to respond to thymus-dependent antigens and were thought to respond to thymus-independent antigens by mounting predominantly an IgM response (35). Results of studies have shown that athymic BALB/c mice can produce IgG in response to the murine respiratory pathogen *Mycoplasma pulmonis* (34) and in response to the T-cell-independent antigen α(1→3) dextran (35). In the former study IgG1 was produced and in the latter study all IgG subclasses were produced (apparently T-cell independent) in response to the antigenic stimuli used. The difference in ability to mount an IgA immune response at the gut mucosal level may explain why the *nu*/*nu* mice are more susceptible to campylobacteriosis in our gnotobiotic mouse model. We previously reported (43, 44) differences between *nu*/*nu* and +/*nu* mice in the viable populations of *Campylobacter* spp. present in their intestinal tract, MLNs, and internal organs which also suggests a possible role for thymus-matured T cells in controlling the systemic spread of *C. jejuni* strains from the murine gastrointestinal tract.

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