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Significance of Flagella in Colonization Resistance of Rabbits Immunized with *Campylobacter* spp.

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Cross-protection among different Lior and Penner serogroups of *Campylobacter* spp. was studied. Rabbits were orally immunized by gastric feeding with *Campylobacter* spp., and 27 to 30 days later, they were challenged with matched or unmatched serogroups by the removable intestinal tie adult rabbit diarrhea (RITARD) procedure. When immunized animals were challenged with different Lior serotypes, no protection against colonization was seen; however, when challenged with homologous Lior serogroups, protection was demonstrated. Immune animals were colonized for an average of 1 day or less versus at least 6 days for nonimmune animals. Rabbits challenged with matched Penner-unmatched Lior strains showed only marginal protection. Our study also demonstrated that flagella are important in initiating colonization and eliciting protective immunity. *Campylobacter coli* VC167B3, an isogenic, nonflagellated mutant, did not colonize rabbits regardless of the route of administration. Single feeding of the mutant strain did not protect the host, whereas three feedings, 48 h apart, resulted in complete protection against the flagellated parent strain. When mutant strain immunized rabbits were challenged with other strains of the same Lior serotype, marginal protection was obtained. Immunogold labeling indicated that there is one or more antigens on the cell surface of the nonflagellated mutant which reacts with a polyclonal antiserum from organisms of the same Lior serogroup. These data implicated the flagellum as the cross-strain protective component of the Lior antigen complex.

Available data suggest that vaccination for campylobacter enteritis is possible (6, 15). In both humans and rabbits, specific mucosal anti-*Campylobacter* immunoglobulin A (IgA) antibody levels rise rapidly after oral immunization and challenge (10, 35). Studies of the immune response of a cohort of 111 newborn infants during intestinal infections of enteric *Campylobacter* spp. showed that nearly all of the children were naturally immunized by the age of 2 years (23). American adult volunteers challenged with *Campylobacter jejuni* developed serum antibodies and were protected from subsequent illness, but not against infection with the same strain (7). Prior infection with *C. jejuni* in infant pathogen-free *Macaca nemestrina* monkeys protects against rechallenge (29). Similar results have been seen in other animal studies as well (1, 2, 9). Although resistance to rechallenge has been associated with rapid clearance in ferrets, shedding may persist without clinical signs (5).

Protective immunity is not seen when the challenge strain is different from the strain used to immunize the host. For example, Burr et al. (9) and others (28) have shown that resistance to colonization in rabbits is obtained after rechallenge with the same strain but not with random strains. Recently, Abimiku et al. (1–3) demonstrated an association of the Lior (20), but not the Penner (27), serotype with the ability to protect infant mice against gastrointestinal colonization with different *C. jejuni* strains.

Campylobacter flagellin is the immunodominant antigen recognized during an infection and is an important virulence factor during colonization (6, 11, 19, 24, 25, 34). However, there are questions of whether the protection is elicited only by the immunodominant flagella or by other somatic antigens as well. The Lior serotype (20) is based on the absorption of hyperimmune antisera with homologous heat-stable antigens followed by absorption with heterologous heat-labile antigens. Thus, the remaining Lior serotype antibodies are very likely not only elicited by flagellin but also by surface antigens. Therefore, in the case of the Lior antigen, we may not be dealing with a single antigen but probably with an antigen complex of which flagellin is a component. A number of investigators (8, 25) have reported that some of the Lior antigens may be expressed on the surface of bacteria both in flagellar and aflagellar strains.

Recent progress in understanding the composition and production of the flagellum and its genetic basis enables us to evaluate further the importance of flagellar antigens in colonization resistance. To accomplish this, we first established the significance of serotypes in predicting resistance to colonization. Then, by using a nonflagellated isogenic mutant (16), we studied the role of flagella in predicting cross-protection among *Campylobacter* strains. We obtained evidence that the flagellum is the cross-strain protective component of the Lior complex.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are shown in Table 1. Bacterial suspensions for oral immunizations (gastric feeding) and the removable intestinal tie adult rabbit diarrhea (RITARD) procedure were prepared, with minor modifications, as previously reported (9, 12). Briefly, the different *Campylobacter* strains were stored as frozen stocks at −70°C. The cultures were thawed, inoculated on selective campylobacter blood agar plates (Remel, Lenexa, Kans.), and incubated overnight at 37°C in plastic bags under campylobacter gas (5% O_2, 10% CO_2, 85% N_2) (Air Products, Allentown, Pa.). The bacteria were then suspended in brucella broth supplemented with 0.04% cysteine and 0.25% serine to an optical density at 625 nm of 0.05 to 0.07. Eight milliliters of the suspension was overlaid in

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TABLE 1. *Campylobacter* strains

Strain[a]	Species	Serotype		Source or reference[b]
		Lior	Penner	
HC	*C. jejuni*	16	27	9
VC74	*C. jejuni*	11	18	21
VC87	*C. jejuni*	1	4	21
VC89	*C. jejuni*	2	1	21
VC91	*C. jejuni*	11	23/53	Lior
VC95	*C. coli*	7	13/16/50	Lior
VC106	*C. jejuni*	2	1	21
VC108	*C. jejuni*	17	4	Penner
VC156	*C. jejuni*	8	28/11	22
VC159	*C. jejuni*	8	28/11	22
VC167	*C. coli*	8	28	22
VC167B3	*C. coli*	8	28	16
81116	*C. jejuni*	6	6/7	26

[a] All strains except HC were isolated from patients' feces. HC is a blood isolate.

[b] VC strains were isolated by H. Lior or J. L. Penner and were received from T. J. Trust's laboratory.

25-cm^2 tissue culture flasks (Becton Dickinson Labware, Oxford, Calif.) containing 5 ml of brucella blood agar and incubated ungassed at 37°C. After overnight growth (1:10 dilution with an optical density at 625 nm of 0.190 to 0.210), the bacteria were pooled and used for oral immunizations or the RITARD procedure (12, 30, 33). One milliliter of the suspension contained approximately 5×10^9 CFU.

Animal study protocol. Female New Zealand White rabbits (Hazelton Research Products, Denver, Pa.) were used in the study. Animals were held for at least 7 days for quarantine and acclimatization in a special holding area before use in experiments. Four to five rabbits were used per group. Food was withheld 18 to 24 h prior to each procedure; however, water was provided ad libitum. Rabbits (0.9 to 1.1 kg) were orally immunized (gastric feeding; 15 ml) as previously described (9, 13), and then 27 to 30 days later, they were challenged (10 ml; challenge doses varied between 35×10^9 and 75×10^9 CFU per rabbit) by the RITARD procedure (12, 30, 33). The weights of the rabbits at the time of challenge were 1.8 to 2.2 kg.

Following gastric feedings and the RITARD procedure, colonization of rabbits was monitored by daily rectal swabs. The swabs were plated directly onto campylobacter blood agar plates for culture under campylobacter gas at 37°C for 48 h. *Campylobacter* spp. were identified by a positive oxidase test (Oxidase Reagent Droppers; Marion Scientific, Kansas City, Kans.) and by microscopic examination. An animal was considered to be free of *Campylobacter* spp., i.e., not colonized, after four consecutive days of negative cultures. Even though every attempt was made to standardize the experiments, the colonization time of nonimmunized rabbits occasionally varied from one group to another (see Table 4). Thus, each experiment included its own control group.

Since previous results (9) and our own findings (data not shown) indicated that the highest levels of IgA antibodies in intestinal secretions were found 5 to 8 days postimmunization and post-RITARD procedure, lavage was performed as previously described (9, 10) on fasted rabbits 7 days after the respective procedure. Briefly, 4 doses (15 ml each) of lavage electrolyte fluid were administered via a gastric feeding tube followed by an intraperitoneal dose of pilocarpine. The fluid stool was collected, crudely filtered through gauze, and processed with bacterial and protease inhibitors. The lavage fluid was then frozen until use.

The experiments reported herein were conducted according to 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 (NIH) publication 86-23, 1985.

Detection of mucosal IgA. IgA antibody levels present in lavage fluids were measured in 96-well microtiter plates by enzyme-linked immunosorbent assay (ELISA; 14). Plates were coated with different antigenic preparations, including whole cells, sonicated whole cells, crude sheared flagella, or glycine extracts (21). Glycine extract antigenic preparations resulted in optimal IgA detection and were used exclusively in the present study. The ELISA procedure involved incubating the plates with 0.1 ml per well of 1.0 μg of glycine extract per ml at 37°C for 1 h and at 4°C overnight. Plates were washed and blocked with a 0.1 Tween-20–5% bovine serum albumin solution. Samples from multiple rabbits within a single experiment were pooled by treatment, and pooled lavage from multiple experiments was used for ELISA. Lavage, preprocessed and filtered (9, 10), was appropriately diluted, and 0.1 ml per well was incubated at 37°C for 2 h. The plates were washed, and 0.1 ml per well of horseradish peroxidase-conjugated, goat anti-rabbit IgA was incubated at 37°C for 30 min. Chromatographically purified, monospecific anti-IgA was prepared in-house and commercially conjugated (Kirkegaard and Perry, Inc., Gaithersburg, Md.). The plates were washed, and 0.1 ml per well of ABTS [2,2'-azino-di(3-ethyl-benzthiazoline sulfonate)] mixed with an equivolume of 5% hydrogen peroxide was added. The substrate reaction was read spectrophotometrically at a wavelength of 414 nm after a 1-h incubation at 23°C. Wells not coated with antigen were used to zero the optical reader, and negative control lavages were included to determine the background. The end point titer was considered to be 3 standard deviations (SD) above the negative controls.

Immunoelectron microscopy. Samples were prepared from overnight growth in biphasic culture media (5% brucella agar and brucella broth) at 35 to 37°C. Broth cultures were centrifuged for 10 min, and the pellet was resuspended in phosphate-buffered saline (PBS), pH 7.6. Immunoelectron microscopy was done as previously reported (18). Live bacteria were incubated with antiserum in PBS at 4°C for 60 min. After three washings in PBS, samples were incubated with goat anti-rabbit IgG linked to 10-nm colloidal gold (Sigma Chemical Co., St. Louis, Mo.) at 4°C for 40 to 60 min and fixed in 2% glutaraldehyde–1% paraformaldehyde for 30 min. Antibody-labeled samples were examined directly on Formvar-carbon-coated grids. The same sample was also postfixed in 1% osmium tetroxide for 30 min and processed for transmission electron microscopy by standard methods. The polyclonal Lior 8 antiserum prepared against *C. coli* 52 used in the immunogold labeling experiments was kindly provided by H. Lior.

RESULTS

Intestinal colonization of orally immunized rabbits following RITARD challenge. Preliminary work has shown that orally immunized rabbits are resistant to recolonization with the same strain but are susceptible to other strains (9). We have extended this work to include a number of different strains (Table 1) of matched and unmatched Lior (20) and Penner (27) serotypes. Rabbits orally immunized with *C. jejuni* VC159 (Lior 8 and Penner 28/11) or VC89 (Lior 2 and Penner

TABLE 2. Colonization of orally immunized rabbits following RITARD challenge with matched Lior and Penner serotypes

Immunization			Challenge			No. of days of colonization (mean ± SD)
Strain	Serotype		Strain	Serotype		
	Lior	Penner		Lior	Penner	
VC159	8	28/11	VC159	8	28/11	0.8 ± 1.3
None			VC159	8	28/11	7.2 ± 2.6
VC159	8	28/11	VC159	8	28/11	<1.0
VC159	8	28/11	VC156	8	28/11	0.2 ± 0.4
None			VC156	8	28/11	6.0 ± 1.8
VC89	2	1	VC89	2	1	1.0 ± 1.4
VC89	2	1	VC106	2	1	2.4 ± 1.1
None			VC106	2	1	>8.0

1) were RITARD challenged with the same and different strains having identical serogroups. After the RITARD procedure, these immunized rabbits shed the organisms for only about 24 h compared with nonimmunized controls, which continued to give positive results for over 6 days (Table 2). Because the animals were examined at 24-h intervals, rather large SD were frequently seen. For example, when VC159-immunized rabbits were rechallenged with the same strain (Table 2), three rabbits were continuously negative, one was positive at 24 h, and one was positive for 3 days, resulting in an SD of 1.3 days. It should also be noted that, since the first rectal swab was taken 24 h after challenge, for groups in which all the animals were negative at 24 h as well as for the subsequent swabs, the length of colonization was reported as <1.0 day rather than 0 days.

When immunized rabbits were challenged with a strain which had different Lior and Penner serotypes, there was no resistance to colonization (data not shown). The results clearly indicate that a single gastric feeding elicits resistance to colonization by strains with homologous serotypes.

Similar results were obtained when immunized rabbits were challenged with organisms of matched Lior but unmatched Penner serotypes (Table 3). The difference in the duration the organisms colonized the immunized and nonimmunized rabbits was about 6 days. However, when immunized rabbits were challenged with strains of the same Penner but a different Lior serotype, the results were not as definitive. The data from two such experiments are shown in Table 4. In experiment 1, the animals received the usual challenge dose (approximately 50×10^9 CFU per rabbit).

TABLE 3. Colonization of orally immunized rabbits following RITARD challenge by matched Lior and unmatched Penner serotypes

Immunization			Challenge			No. of days of colonization (mean ± SD)
Strain	Serotype		Strain	Serotype		
	Lior	Penner		Lior	Penner	
VC159	8	28/11	VC159	8	28/11	0.4 ± 0.5
VC159	8	28/11	VC167	8	28	<1.0
None			VC167	8	28	7.2 ± 1.3
VC74	11	18	VC74	11	18	1.2 ± 1.1
VC74	11	18	VC91	11	23/53	0.8 ± 0.8
None			VC91	11	23/53	6.2 ± 1.8

TABLE 4. Colonization of orally immunized rabbits following RITARD challenge with unmatched Lior and matched Penner serotypes

Expt	Immunization			Challenge[a]			No. of days of colonization (mean ± SD)
	Strain	Serotype		Strain	Serotype		
		Lior	Penner		Lior	Penner	
1	VC87	1	4	VC87	1	4	1.2 ± 1.6
	VC87	1	4	VC108	17	4	2.8 ± 1.3
	None			VC87	1	4	5.0 ± 2.0
2	VC87	1	4	VC87	1	4	<1.0
	VC87	1	4	VC108	17	4	6.0 ± 1.7
	None			VC87	1	4	12.0 ± 0.8
	None			VC108	17	4	12.0 ± 0

[a] In experiment 1, the challenge dose was ca. 50×10^9 CFU per rabbit, whereas in experiment 2, the dose was ca. 50×10^3 CFU per rabbit.

Although a difference was seen between the two groups, it was less substantial. The colonization time for the unmatched group was 2.8 ± 1.3 days, whereas for the control group, it was 5.0 ± 2.0 days, perhaps indicating some degree of protection against colonization. Since the challenge dose used in this experiment was relatively high, it is possible that the host's defensive mechanisms may have been overwhelmed, obscuring any reduced levels of protection. When the challenge dose was reduced by 6 $\log_{10}$ to 10^3 CFU per rabbit, marked differences in colonization time were observed between the immunized and nonimmunized groups (Table 4, experiment 2). As pointed out in Materials and Methods, different groups of rabbits may vary in their ability to clear the organisms. This is probably due to the biological variability of the rabbits (Table 4, experiment 2). Nevertheless, there was a significant difference between the immunized and nonimmunized animals. However, the protection was considerably greater when both the Lior and Penner serotypes were matched. Thus, the results suggest that only limited protection may be elicited by the Penner antigen.

Lack of cross-protection among strains with related flagellar sequences. Since randomly selected *Campylobacter* strains with unmatched Lior antigens did not confer protection, we tested strains with genetically related flagella. Thornton et al. (31) constructed DNA probes from different regions of the flagellin genes of *C. coli* VC167 and used these probes to examine nucleotide sequence similarities among 30 strains of 20 serogroups. The results indicate that *Campylobacter* flagellin genes are highly conserved at the 5′ and 3′ ends and show varying degrees of sequence similarity in the central region. This allowed us to select strains with similar flagellin genes for the cross-protection studies. Four different strains, Lior 8 (VC159) versus Lior 7 (VC95) and Lior 8 (VC167) versus Lior 6 (81116), were studied. Challenge doses of 10^9 and 10^3 CFU per rabbit were tested. With neither dose was there any suggestion of cross-protection against colonization (data not shown). These results indicate that the sequence homology of flagellin was not, in this case, enough to provide immune protection. Complete and effective protection is elicited only against the flagellin of the same Lior serotype.

Localization of Lior antigens. Immunogold-labeled Lior 8 serogroup polyclonal antiserum made against *C. coli* 52, biotype I (Lior), randomly labeled the shaft of the Lior 8 serotype *Campylobacter* (VC167, biotype II) flagella and the cell surface (Fig. 1), but it did not react with Lior 7 serotype strains (data not shown). The labeled antiserum also reacted

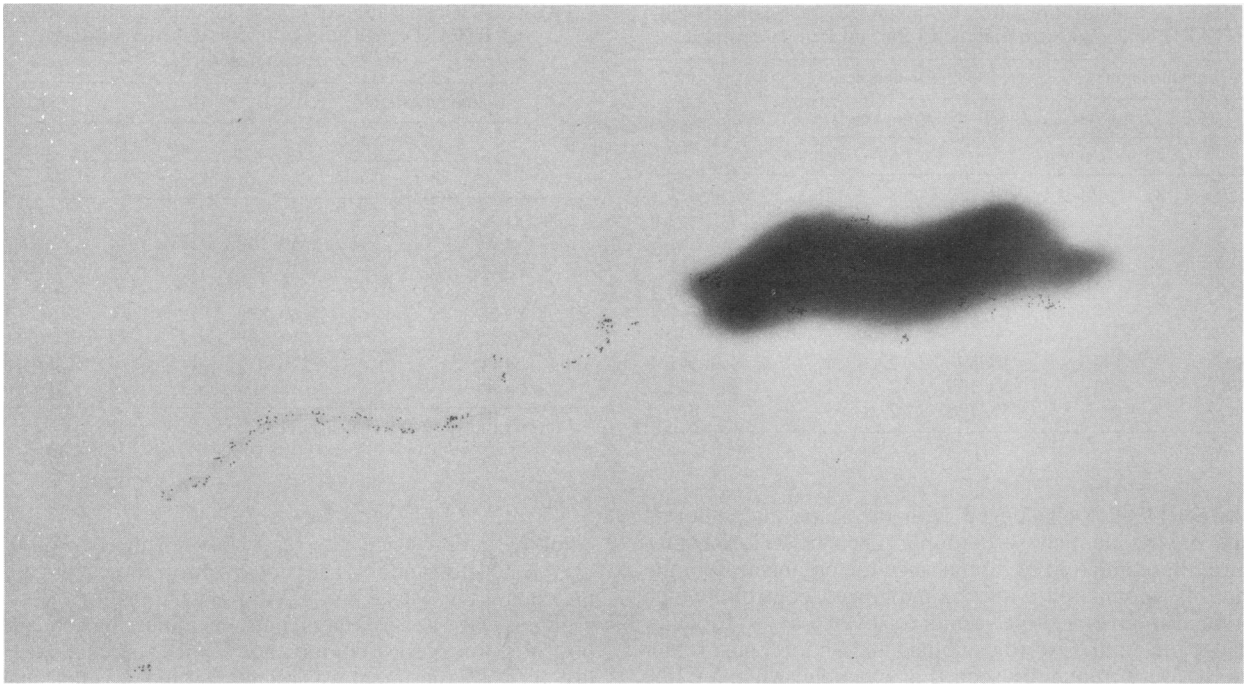

FIG. 1. Immunogold electron micrograph of *C. coli* VC167 (Lior 8) against Lior 8 polyclonal antiserum (*C. coli* 52), showing antigenic specificity outlining the randomly labeled flagellar shaft and cell surface. Magnification, ×25,000.

with the somatic antigens of the Lior 8 aflagellar mutant (VC167B3) (data not shown). These visual observations are consistent with the results of our cross-protection studies between homologous and heterologus strains (Tables 2 through 5) as well as with the data obtained by Harris et al. showing the presence of Lior-specific antigens of the surface of VC167 (17). The homologous strains, including nonflagellated strains, share common antigens, whereas strains of different Lior serotypes do not.

The role of flagella in colonization. Reproducible and effective protection against colonization associated with the Lior serogroup antigens, of which flagella appear to be a component (2, 4, 7, 25, 26, 32), suggests that flagella may be important in colonization of the host. To examine this question more directly, we used a kanamycin-resistant, isogenic nonflagellated deletion mutant (VC167B3) developed by Guerry and coworkers (16) from a flagellated strain (VC167). The nonflagellated mutant, for all practical purposes, did not colonize rabbits. Whether the dose was administered by gastric feeding or the RITARD procedure, the mutant colonized the animals for less than 24 h, whereas the parent colonized the host for several days. In the case of gastric feeding, the average colonization by the parent strain was 5.4 ± 0.9 days; for the RITARD procedure, it was 4.6 ± 0.9 days. On those rare occasions when the mutant was reisolated from the rabbits, the organisms were kanamycin resistant and nonmotile.

TABLE 5. Colonization of rabbits orally immunized with a nonflagellated mutant (VC167B3) and RITARD challenged with either the parent strain (VC167) or a strain of an homologous Lior serotype (VC159)

Expt	Immunization		Challenge[a]	No. of days of colonization (mean ± SD)
	Strain	No. of doses	Strain	
1	VC167B3	0	VC167	6.7 ± 2.1
		1	VC167	7.5 ± 1.7
		3	VC167	1.6 ± 1.5
2	VC167B3	0	VC159	6.2 ± 1.5
		3	VC159	6.6 ± 0.9
3	VC167B3	0	VC159	>8.0
		3	VC159	2.0 ± 2.1
		0	VC167	>8.0
		3	VC167	0.5 ± 0.6

[a] RITARD challenge doses for experiments 1 and 2 were approximately 50 × 10^9 CFU per rabbit, whereas for experiment 3, it was approximately 50 × 10^3 CFU per rabbit.

Since VC167B3 did not readily colonize rabbits, multiple (three) gastric feedings of the mutant were given 48 h apart to enhance any potential immune response. The immunizations were followed by RITARD challenge with matched (Table 5) or unmatched (data not shown) Lior serotypes. Rabbits receiving three gastric feedings were protected against the parent strain, whereas a single dose immunization did not protect the host (Table 5, experiment 1). Multiple immunizations did not protect against a nonparent homologous Lior strain (Table 5, experiment 2). Preliminary studies, however, indicate that when the challenge dose of the nonparent strain of the homologous serotype is decreased by 6 logs to 10^3 CFU per rabbit, partial protection occurs (Table 5, experiment 3). These data indicate that, although flagella may be necessary for colonization as indicated in the previous experiment, other bacterial antigens can induce colonization resistance in the host (Table 5).

Anticampylobacter intestinal IgA response after primary and secondary challenge. IgA antibody levels in intestinal lavage from rabbits challenged with the parent (VC167) or

the nonflagellar mutant VC167B3 were determined by ELISA against glycine extracts of both strains. Rabbits which were only fed the bacteria developed no significant IgA titers, with end point titers of <10. On the other hand, nonimmunized (not orally fed) rabbits that were challenged by the RITARD procedure developed slightly higher levels of IgA. IgA titers with VC167 and the mutant (VC167B3) were measured at 400 and 40, respectively. However, significant levels of IgA antibodies were detected in immunized rabbits (VC167 or VC167B3) after RITARD challenge with VC167. The highest levels of mucosal IgA were obtained when the animals were immunized with the homologous parent strain rather than the mutant. End point titers in the range of 34,000 were obtained from rabbits immunized with VC167, and end point titers were between 1,000 and 4,000 when immunized with the nonflagellated mutant (VC167B3).

DISCUSSION

We have shown that the Lior serotype can be used to predict cross-protection among *Campylobacter* strains after active immunization of adult rabbits. Rabbits inoculated orally with various *C. jejuni* and *C. coli* strains became resistant to colonization by organisms of the same Lior serotype, as determined by culture of rectal swabs after secondary challenge by the RITARD procedure (Tables 2 and 3). The ability of the Lior serotype to predict cross-strain protection was apparently due to the flagellum associated with the Lior antigen complex. When rabbits immunized with the nonflagellated strain, VC167B3, were challenged with different strains of the same Lior serotype as its parent (VC167), no significant protection of the immunized animals was seen. However, with marginal infective challenge doses (approximately 50×10^3 CFU), some protection was observed (Table 5, experiment 3). These data suggest that within the same Lior serotype a major common protective antigen resides in or on the flagella, but a less-effective common antigen(s) may be located on the cell surface.

Protection associated with Lior antigens is not extended to different Lior serotypes even if they have related flagellar sequences. When we challenged immunized rabbits with heterologous Lior strains, which, based on DNA homology (31), had similar flagellar segments, no protection was seen. Although the reason for this is not known, it may be that these regions are not immunogenic, are not exposed during infection due to tertiary protein structure, or are not a part of the functional segments of the flagella involved in colonization. Alternatively, antibodies directed against flagellar protein may not all be protective. Newell (25), for example, has reported that of eight monoclonal antibodies derived from mice hyperimmunized with purified *C. jejuni* flagella, none protected infant mice against colonization with the parent.

The present study established that flagella are necessary for colonizing experimental animals. When an isogenic aflagellar deletion mutant (VC167B3) was fed intragastrically or injected directly into the mid-small bowel of a rabbit, it was cleared within 24 h with no significant IgA response. *Campylobacter* strains possessing intact flagella typically colonized rabbits for 4 to 12 days. The inability of the nonflagellar strains to colonize the host is also reflected by the lack of protection following a single feeding (Table 5, experiment 1) and insignificant levels of intestinal IgA.

Though the Lior serotype antigens appear to be immunodominant and effective in producing protective antibodies, it is obvious, as indicated above, that antigens not located on flagella also contribute to eliciting such protection in an infected host. IgA specific for nonflagellar antigens was measured against glycine extract of VC167B3 (21). Titers remained elevated compared with those obtained against glycine extract of the flagellar parent (VC167), indicating significant levels of IgA against nonflagellar antigens. We have identified by immunogold labeling multiple antigenic sites on the flagella and the cell surface of strain VC167 and also on the cell surface of its nonflagellar mutant (VC167B3) (data not shown). Similar observations with monoclonal antibodies have been made by Newell (25). The extent to which these somatic and flagellar antigens are involved in producing protective antibodies is not clear. Although a single feeding of the nonflagellar mutant did not protect the rabbit against challenge with the parent strain, three feedings did (Table 5, experiment 1). These data indicate that antigens not located on the flagella can produce protection against homologous flagellated strains. This, of course, does not preclude the role of flagella in colonization, nor does it exclude flagellar immunogenicity.

Although nonflagellar antigens may be involved in eliciting protection against colonization (Table 5), partial protection seen with these minimal infective doses (Table 5) may have been attributable to lipopolysaccharide (LPS), since the immunizing strain (VC167B3) and the challenge strain VC159 have similar, although not identical, Penner serotypes: 28 (smooth-colony type) and 28/11 (rough), respectively (Table 1). The exact similarities or differences between these two LPS serotypes is not known.

Challenges of immunized rabbits with matched Penner and unmatched Lior serotypes suggest that the Penner antigen (*Campylobacter* LPS) (27) also elicits limited protection (Table 4). When immunized rabbits were challenged with standard infective doses (10^9 CFU per rabbit) of unmatched Lior serotypes but matched Penner serotypes, no significant differences in colonization times between the controls and the matched Penner serotype strains (Table 4, experiment 1) were seen. However, when the infective dose was decreased to the minimal level (10^3 CFU per rabbit) (Table 4, experiment 2), the animals were protected against colonization by a matched Penner serotype.

Whether Lior antigen associated with the flagellum is the flagellin protein or an associated protein is not certain from our data. It is possible that an associated protein might be more immunogenic when present on the flagellum than on the cell surface, but experiments with flagellated organisms suggest that it is more likely that the antigen on the flagellum is not the same as that on the cell surface. In this case, it would not be surprising if the Lior antigen which confers strong cross-strain protection is flagellin.

In conclusion, cross-protection among *Campylobacter* strains can only be predicted on the basis of Lior serogroups of flagellated organisms. The nonflagellated mutant (VC167B3), while typeable within the Lior serogroup, has lost Lior serogroup protection capability. Nonflagellar somatic antigens apparently can elicit resistance to colonization but do not impart strong cross-strain protection.

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