INFECTION AND IMMUNITY, Feb. 1986, p. 536–546
0019-9567/86/020536-11$02.00/0

Vol. 51, No. 2

Mucus Colonization as a Determinant of Pathogenicity in Intestinal Infection by *Campylobacter jejuni*: A Mouse Cecal Model

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Received 23 August 1985/Accepted 21 October 1985

Human isolates of the intestinal pathogen *Campylobacter jejuni* have been shown to colonize mucus on the outer surface and deep within the intestinal crypts of gnotobiotic or germfree mice. The cecal crypts are preferentially colonized. A model of mucus colonization by *C. jejuni* in the mouse cecum has been developed, using antibiotic- and magnesium sulfate-treated specific-pathogen-free animals. These spiral-shaped bacteria colonize the mucus in a similar manner to the normal spiral-shaped microbiota. No evidence of adhesion to the intestinal surface was found with a wide variety of microscopic techniques. The campylobacters were seen to be highly motile in living preparations of gut tissue and rapidly tracked along intestinal mucus. Just as many of the normal spiral-shaped bacteria of intestinal surfaces can achieve close association with the epithelium through mucus association and do not adhere to the surface, *C. jejuni* colonizes the intestinal mucosa via mucus colonization. Thus, a major determinant of pathogenicity in intestinal infection with *C. jejuni* is proposed to be an ability to colonize intestinal mucus. The possession of specific adhesins is unlikely to be a significant determinant of pathogenicity. Better understanding of the mechanism of mucus association and the properties of the bacterium that are responsible will provide a basis for the rational selection of preventative measures. The model of mucus association in adult antibiotic-treated mice provides an opportunity for colonization studies with variant organisms and immunization studies.

The intestinal surfaces of the majority of animal species are normally populated by large numbers of bacteria (6). Over the period of evolution these organisms have acquired a wide range of sophisticated adaptations which allow them to survive in the many ecological niches provided by the gut surface. To remain associated with the mucosa, these bacteria need to withstand the flow of the intestinal chyme. Three mechanisms of association have evolved: (i) adhesion, in which bacteria attach to the epithelium by means of specific adhesins (20) or development of specialized insertion structures (2); (ii) surface mucus colonization, in which certain organisms have the ability to survive in and presumably multiply in the outer areas of the mucus layer covering the gut surface (13); and (iii) deep mucus and crypt association, in which the mucus-filled crypts of Lieberkuhn and crypts of the large bowel are colonized in most animal species by dense aggregations of bacteria (11).

Given that the first stage of the disease process in most intestinal infections is association with the gut surface by the pathogen and that pathogens have also evolved with their host, it is reasonable to expect that these organisms will have acquired mechanisms of association similar to those of the normal microbiota. Adhesion of intestinal pathogens to the mucosa has certainly been well proven and the search for specific adhesins has intensified in recent years (15). However, adaptation to a mucus environment as a mechanism of association with the mucosa by gut pathogens has been ignored, although the early work of Freter and co-workers suggested that this might be important for *Vibrio cholerae* infection (4).

The working hypothesis used in the current work was that *Campylobacter jejuni* was a likely pathogen to be a mucus and crypt colonizer. The spiral morphology and microaerophilism of this bacterium were similar to the normal inhabitants of intestinal crypts. Therefore, germfree and gnotobiotic mice were fed pure cultures of human isolates of this organism. Following the success of these experiments, as described below, a more practical model of mucus colonization was developed, using antibiotic-treated specific-pathogen-free (SPF) animals.

Scanning electron micrographs of *C. jejuni* in baby mouse intestine have been cited as evidence of adhesion playing a role in pathogenesis (3). Development of the mouse model has allowed a closer examination of the association of this organism with the gut surface. By using a combination of microscopic techniques, including videotaping of fresh wet preparations of intestinal mucosae, it has been shown that *C. jejuni* associates with intestinal tissue by mucus colonization and that adhesion is unlikely to be an important factor.

MATERIALS AND METHODS

Bacterial cultures. The following strains of *C. jejuni*, both isolated from proven cases of campylobacter enteritis in humans, were used: strain 200 from the Royal Alexandra Hospital for Children, Camperdown, N.S.W., Australia; Strain Cj.Vic. from a large outbreak on a university campus.

Animals. Germfree BALB/c mice were obtained from the Australian Atomic Energy Commission animal house, Lucas Heights, N.S.W. Gnotobiotic animals were mice from the same source which had been given pure cultures of a limited number of isolates from SPF mice and maintained in a flexible plastic isolator and fed sterile food and water. These isolates were a *Lactobacillus* sp., a *Bacteroides* sp., and a *Fusobacterium* sp. The mouse strain used in Victoria was the CD1 strain of SPF animals from Charles River Canada Ltd., Montreal, Que. The so-called spiral-free animals from a colony of SPF BALB/c mice which were initially derived from germfree stocks and had been maintained for several

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years under strict barrier conditions. A normal microbiota had developed in these animals that lacked any surface-associated spirals.

After inoculation of the *Campylobacter* sp. cultures, all animals were maintained in a laminar-flow clean working area. Uninoculated control animals were always maintained under the same conditions to ensure that no spiral organisms of the normal microbiota had established.

Preparation of cultures for inoculation. Overnight cultures of *C. jejuni* on lysed blood agar (7.5% horse or human group O blood in blood agar base no. 2 [Oxoid]) grown in a microaerophilic environment (BBL Microbiology Systems jar with anaerobic generator sachet and no catalyst) at 37°C were harvested in tryptone soy broth (Oxoid). The concentration of organisms was adjusted to approximately 10^9 per ml.

Animal inoculation. Adult mice were lightly anesthetized with ether, and 0.1 ml of a 5% solution of sodium bicarbonate was administered directly into the stomach via 0.86-gauge plastic tubing (Portex, Boots Catalog PP100) followed by 0.1 ml of the bacterial suspension.

Magnesium sulfate treatment. Mice were lightly anesthetized with ether, and 0.1 ml of a saturated solution of $MgSO_4$ was administered directly into the stomach via the plastic tubing.

Antibiotic treatment. All antibiotics were given ad lib in the drinking water for the period given in the text. The following antibiotics and concentrations were used: vancomycin (Sigma Chemical Co.), 0.05 g/100 ml; kanamycin (Sigma), 0.1 g/100 ml; ampicillin (Sigma), 0.1 g/100 ml.

Experimental protocol for mucus colonization of antibiotic-treated mice by *C. jejuni*. Below is a detailed protocol of the method finally selected for producing SPF mice with cecal mucosae colonized with *C. jejuni*: days 1 and 2—kanamycin, ampicillin, and vancomycin, animals on food; days 3 and 4—vancomycin, animals on food; day 5—vancomycin, animals off food, $MgSO_4$ administered at 4 p.m.; day 6—vancomycin, animals off food, $MgSO_4$ administered at 9 a.m., 12 noon, and 2 p.m., *C. jejuni* given at 4 p.m., animals put back on food; day 7—vancomycin, animals on food, *C. jejuni* given at 9 a.m.; day 8—animals sacrificed.

Histological methods. Animals were killed by spinal dislocation, and 0.5-cm lengths of the distal ileum, cecum, and colon were removed and then fixed and embedded as previously described (11). Thick sections (0.5 μm) and thin sections (50 to 70 nm) were cut on a Reichart ultramicrotome.

(i) Light microscopy. Thick sections were stained with methylene blue containing 1% borax and examined under oil immersion.

(ii) Electron microscopy. For scanning electron microscopy (SEM), pieces of the fixed tissue were critical-point dried, placed on stubs, sputter coated with platinum (Dynavac), and examined with a Philips 505 SEM. For transmission electron microscopy (TEM), thin sections were made of selected tissue, stained with uranyl acetate and lead citrate, and then examined with a Philips 300 TEM. For inverted back-scatter scanning, thick sections (0.5 μm) were air dried onto gold palladium (300 nm)-coated cover slips and stained with uranyl acetate for 1 h and lead citrate for 2 min as for TEM. The sections were examined with a Jeol 35C SEM, using a Robinson detector. This is a scintillation-type back-scatter detector which detects the back-scattered electrons which penetrate into specimens deeper than secondary electrons; they are also capable of discriminating between different atomic numbers. Thus, the technique picks up the stains osmium tetroxide, uranyl acetate, and lead citrate, all of which have high atomic numbers. The polarity of the microscope is reversed to give a reverse image which is comparable to images seen under the light microscope.

Assessment of crypt colonization. Thick sections were cut of the relevant tissues from at least five mice in each experimental group. A co-worker was asked to label these slides and randomly ascribe a number to each slide. One of us (A.L.) then examined each of the more than 250 slides and scored them for crypt colonization, using the following semiquantitative assessment: no spirals in the crypt score = 0, 1 to 15 = 1, 16 to 30 = 2, 31 to 40 = 3, and >50 = 4. Ten to 15 crypts were scored in each section. The code for the slides was then revealed and the results were tabulated. The value "percent crypts colonized" refers to the proportion of crypts that were scored 2 or more, i.e., had more than 30 organisms in them.

Videotaping of wet preparations. Animals were killed by spinal dislocation, and the cecum was removed and opened. The cecal contents were gently cleared from the surface with the back of a scalpel blade. The surface of the tissue was then lightly scraped with the back of a blade so as to remove the mucus and underlying tissue. The scrapings were placed on a slide and a cover slip was placed on top. A tissue was placed over the cover slip which was then pressed down very hard with the thumb. The slide was examined immediately, using an oil immersion objective (×100) under phase-contrast microscopy. Videotapes of the preparation were made on 0.75-in. (1.88-cm) videotape, using a Sony camera and video recorder linked up to the microscope. To get pictures of the crypt-associated campylobacters, the cecal tissue was vigorously washed in tryptone soy broth prior to preparation of the scrapings.

Cultural methods. Whole pieces of intestine were placed in 5 ml of tryptone soy broth and weighed. The tissue was homogenized with a Teflon grinder. Counts were done by using a standardized loop method (17).

RESULTS

Colonization of germfree, gnotobiotic, and "spiral-free" SPF mice. The intestinal mucosa of normal mice is colonized by large numbers of bacteria, i.e., fusiform-shaped bacilli that are seen in the outer mucus blanket and spiral-shaped bacteria that are also seen in the mucus blanket but which also dominate the intestinal crypts. Therefore, conventional animals were unsuitable for studies of mucus colonization by *C. jejuni*. In early experiments a range of animals was available that had no surface-associated spirals: i.e., (i) germfree, a colony of BALB/c germfree mice; (ii) gnotobiotes, an isolator containing some of these BALB/c mice that had been seeded with some pure cultures of mouse intestinal isolates, i.e., a fusiform, a *Lactobacillus* sp., and a *Bacteroides* sp.; (iii) spiral-free, a colony of SPF mice that were maintained under strict barrier conditions in which spiral-shaped bacteria were absent. The latter two groups did have the fusiform bacilli in the outer mucus blanket. These were removed by vancomycin treatment.

The results of a major colonization study of these three groups of mice, using strain 200, are shown in Table 1. No crypts in the small intestine were seen to be colonized by *C. jejuni*. The cecum was the preferred site for the organism, with about 40% of crypts containing large numbers of the characteristic spirals. An example of a crypt from an infected and control animal is shown in Fig. 1. The base of the crypt is packed full with bacteria. Due to the conventional

TABLE 1. Colonization by *C. jejuni* of the intestinal mucosa of mice with no surface-associated microbiota: histological studies

Group	% Crypts colonized		
	Small intestine	Cecum	Large intestine
Germfree	0	41	10
Gnotobiotic[a]	0	40	12
Spiral Free[a]	0	38	9

[a] Vancomycin treated to remove surface-associated "fusiforms."

TABLE 2. Colonization by *C. jejuni* of the intestinal mucosa of mice with no surface-associated microbiota: cultural studies

Group	No. of animals	Concn of *C. jejuni* (log_{10} per g (of tissue and contents)		
		Small intestine	Cecum	Large intestine
Germfree	20	7.5 ± 0.8	9.6 ± 0.1	8.9 ± 0.3
Gnotobiotic[a]	15	7.4 ± 0.8	9.3 ± 0.3	8.8 ± 0.4
Spiral Free[a]	26	7.0 ± 0.7	9.7 ± 0.3	9.0 ± 0.3

[a] Vancomycin treated to remove mucosa-associated fusiforms.

fixation methods used in these studies, much of the surface mucus was lost. However, in the areas of the section where small pieces were retained they were seen to be packed with spirals. Only a small proportion of the colonic crypts were colonized. This preference for the cecum is also shown in the cultural results (Table 2). While the campylobacters can be found in reasonable numbers in the small bowel, the cecum contains very large numbers and is again shown to be the preferred habitat in the mouse gastrointestinal tract, although the colon was also heavily colonized.

Development of a model of mucus colonization by *C. jejuni* in the mouse cecum. The results above show the *C. jejuni* will colonize intestinal mucus in extremely large numbers. A visit of the senior author to a different laboratory without access to germfree animals led to the development of a model of mucus colonization in commercially available SPF mice. This project in itself was considered important as many laboratories do not have the facilities or expertise necessary to maintain animals in isolators. Results of these experiments were more striking than the ones described above.

Conventional mouse colonies and most SPF colonies have a well-established mucosal microbiota. Figure 2 shows the surface-associated microorganisms of the cecum of the Charles River SPF mice used in these studies. The surface is colonized by large numbers of fusiform-shaped bacteria. Mixed with these organisms are spiral-shaped bacteria which

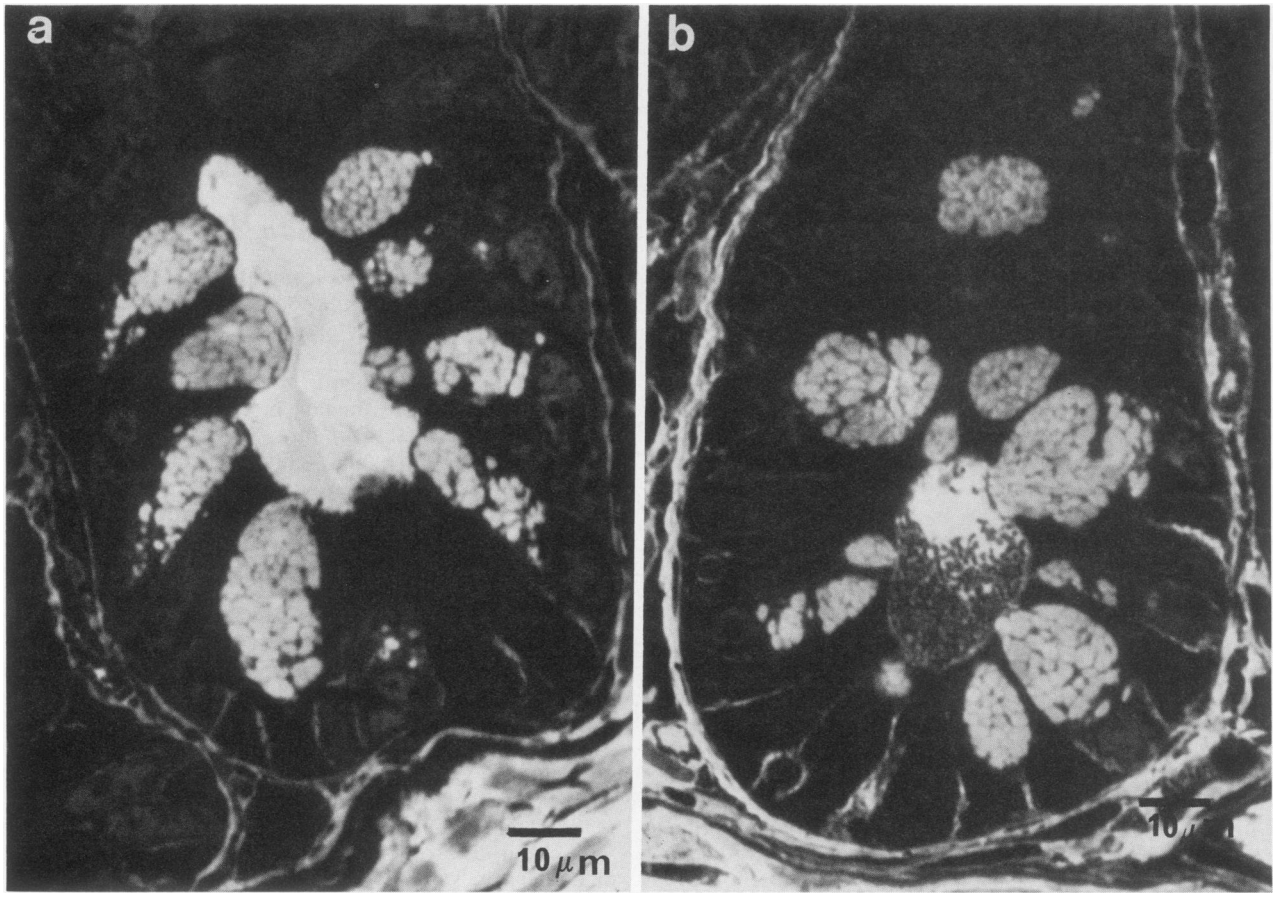

FIG. 1. Cecal crypts from germfree mice. (a) Uninoculated; (b) animal inoculated with a pure culture of a human isolate of *C. jejuni* strain 200. The crypt in the inoculated animal is full of bacteria.

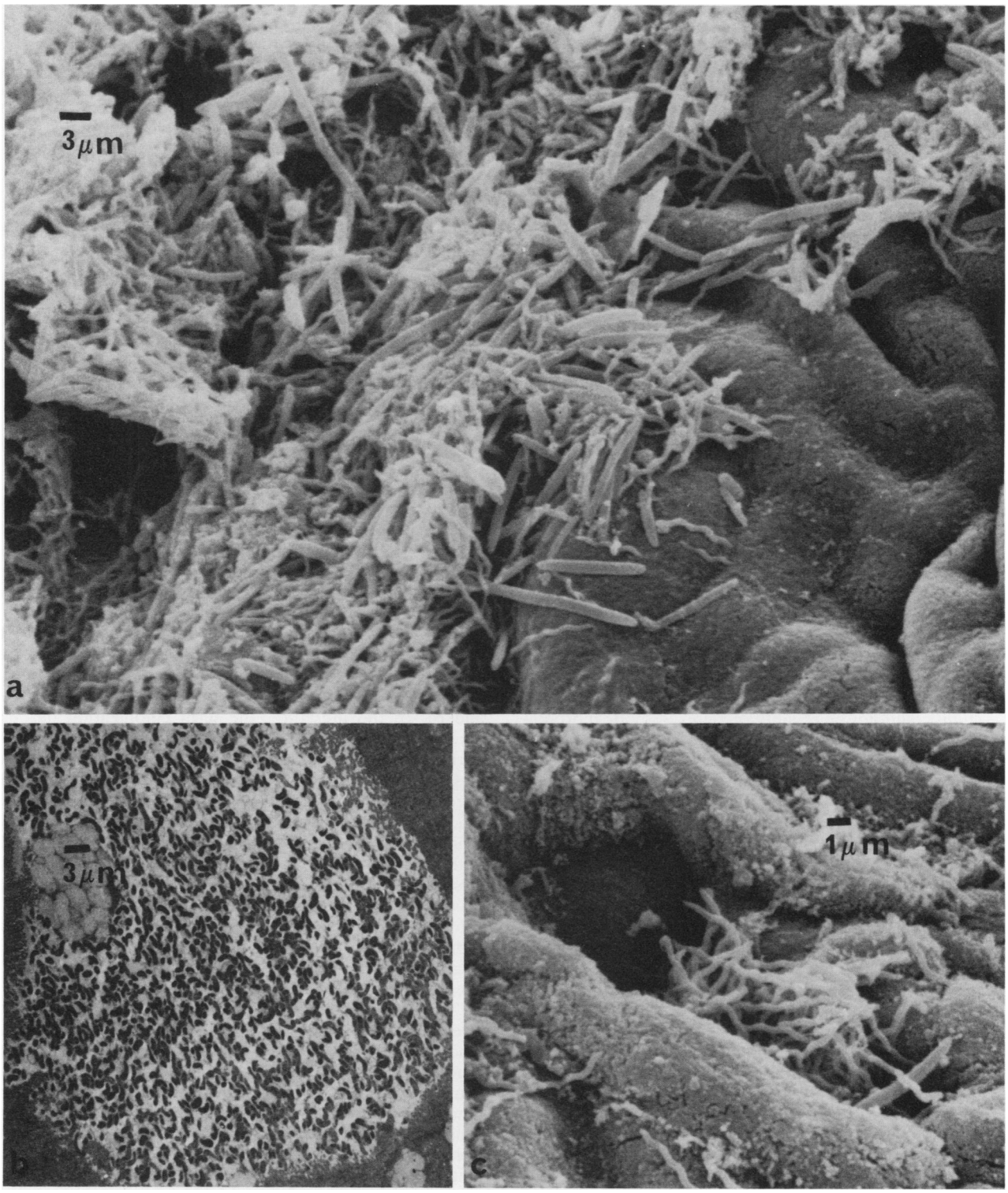

FIG. 2. Cecal mucosa of an SPF mouse showing the surface-associated microbiota. (a) The outer layer is colonized with a variety of bacteria with a fusiform-shaped organism predominating; spiral-shaped bacteria are seen closest to the surface (SEM). (b) A crypt is seen full of bacteria (TEM). (c) The opening of a crypt shows the closely packed spirals (SEM).

are seen to be in highest concentration close to the tissue surface. The spirals continue down into the cecal crypts which are tightly packed with very large numbers of a seemingly "pure" culture. The same picture is found in other animal colonies, although different morphological types of spirals may be found (16). Clearly, these animals could not be used for studies with campylobacter colonization as the organisms are too closely related morphologically

and would not be easily recognized in tissue sections. Also, as the mucus was already so heavily colonized the *C. jejuni* would have difficulty establishing. Therefore, an experiment on colonization with this organism in antibiotic-treated mice was done.

Preliminary studies showed that the spirals could be removed with ampicillin and kanamycin, while vancomycin would remove the fusiform-shaped bacilli. Some groups were treated with magnesium sulfate; previous work had shown that the purging effect of the magnesium sulfate cleared out the crypt organisms and led to increased mucus production (11). In combination with the antibiotics this was likely to provide an ideal environment for the mucus-seeking campylobacters. The results of this experiment with strain Cj.Vic. are shown in Table 3. At least 60 crypts from a total of five mice were examined in each group. Details of the semiquantitative assessment of crypt colonization are given in Materials and Methods. Only the cecal mucosa was examined as it had been shown to be the preferred site of colonization. Cultural studies are not reported for these experiments as the crypt association is a more sensitive measure of mucus colonization than counts of homogenized tissue.

Experiments involving subjective judgments on preparations under the microscope are prone to investigator bias. Special care was taken to eliminate bias in this experiment; the identifying numbers of each slide were masked by a second person. The slides were mixed and each slide was coded. The investigator was not told of the code until all slides had been assessed and the results had been recorded. The results were then tabulated into the experimental groups. These results were remarkably consistent and give us complete confidence in the effectiveness of the antibiotic and magnesium treatments.

C. jejuni could not be reliably recognized by its morphology in these sections, although when the slides were reexamined after the code had been broken differences could be seen: the spirals in the normal animals were longer and thinner than the campylobacters. Thus, the crypt colonization figures for untreated animals with or without *C. jejuni*, as expected, were the same. Either antibiotic treatment alone did not completely eliminate the normal spirals from the crypts or in the time sequence of treatment used they had started to recolonize, as 17% of the crypts had spirals in them. However, 90% of the crypts in the mucosa of these treated animals were heavily colonized with spirals when they had been inoculated with a pure culture of *C. jejuni*. The majority of these spirals had to be the inoculated organism, but the knowledge that some of them must be normal spirals did not make it a totally satisfying model of mucus colonization.

The combination of antibiotic and magnesium sulfate treatments was completely successful in keeping the crypts spiral-free for the period of the experiment. When the code was revealed and the results were looked at, it was impressive that not one of the 68 crypts that had been carefully examined in the animals in this group contained even one spiral-shaped organism. Thus, it can be safely concluded that the organisms that were seen to pack the crypts of identically treated animals which had been inoculated with pure cultures were indeed *C. jejuni*. Some crypts were found to be empty; this was the same as in the germfree and gnotobiotic animals where a proportion of crypts seem to escape colonization.

The tissues from the inoculated antibiotic- and magnesium sulfate-treated animals were then looked at much more closely and by a variety of microscopic techniques to demonstrate this colonization of the mucus layers of the cecal mucosa. Figure 3a shows a low-power shot of a cecal section from one of these animals examined by inverted back-scatter microscopy on an SEM. Three consecutive crypts are seen to be heavily colonized with the spiral-shaped campylobacters. The arrangement of these organisms with a crypt is shown in Fig. 3b and c. The bacteria are arranged in parallel streams similar to that seen in normal animals colonized with natural spirals.

TABLE 3. Colonization of the cecal mucosa of antibiotic- and magnesium sulfate-treated mice with a human isolate of *C. jejuni* strain Cj.Vic.

Group of mice	*C. jejuni*	% Cecal crypts with spirals
Normal	−	98
	+	96
Antibiotic treated	−	17
	+	90
Antibiotic and $MgSO_4$ treated	−	0
	+	88

The very high concentration of organisms seen at the bottom of crypts and the very close proximity to the tissue surface is best illustrated with the TEM (Fig. 4). Some bacteria are so close they appear to be pushing their way through the intestinal cells. Due to difficulty in consistently retaining the mucus blanket, crypt colonization was taken as the measure of mucus colonization. However, where the blanket had been retained, SEM clearly demonstrates the heavy concentration of spirals in the surface mucus (Fig. 5c). SEM also well illustrates the spiral-packed crypts (Fig. 5a and b). In the literature, the term colonization has been taken to describe association with the intestine over a long period. In these experiments association of the campylobacters was only studied over a period of days, as with time the normal spirals returned and so the *C. jejuni* could not be readily distinguished. The preliminary studies made with the limited number of germfree animals available had shown that without competition with the normal flora the campylobacters remained associated with the cecal mucosa for the several weeks the animals were monitored.

Motility of *C. jejuni* in the intestinal mucosa. Examination of fixed cecal tissue from *C. jejuni*-colonized tissue can give a misleading impression. Electron micrographs could be used as evidence of adhesion to the epithelial surface. (Fig. 6). However, based on our experience with the normal intestinal spirals, this appeared unlikely. Rather than being stuck onto the tissue, the campylobacters survive in the mucus and, being highly motile, move around in this environment, sometimes penetrating deep into the crypts. Examination of intestinal scrapings under phase-contrast microscopy had been revealing in our investigation of the normal microbiota. A light scrape of the washed intestinal epithelium, placed on a slide and squashed down hard under a cover slip, retains a degree of tissue integrity; i.e., in some cases whole crypts can be seen. Bacteria remain active for up to 0.5 h in these preparations and so their behavior in intestinal mucus can be investigated. Videotaping allows the motility to be analyzed at leisure.

Scrapings of mucosa from cecal tissue of antibiotic- and magnesium sulfate-treated mice given *C. jejuni* were examined under phase-contrast microscopy and videotaped. The spiral bacterium was highly motile in intestinal tissue, mov-

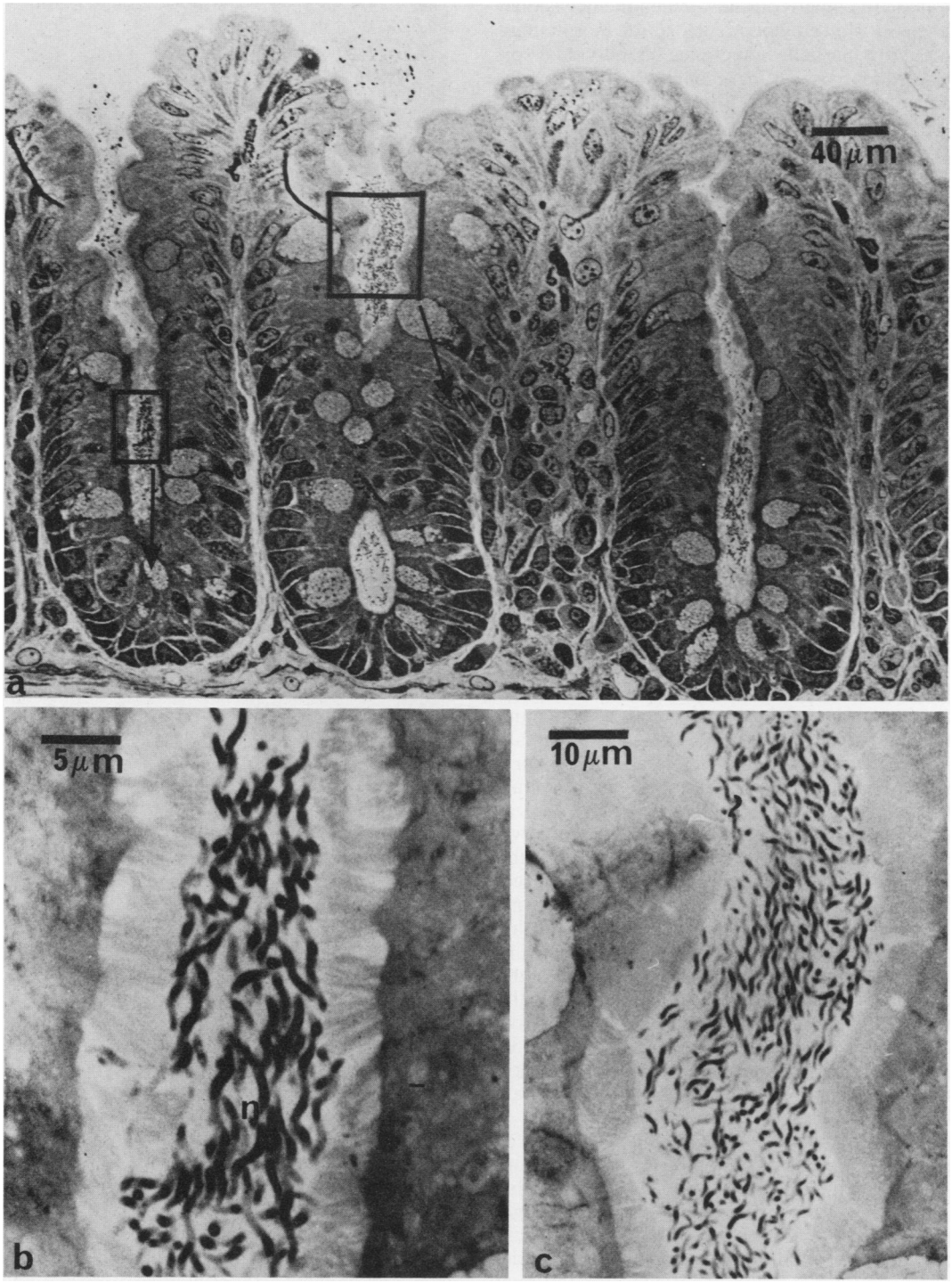

FIG. 3. Cecal mucosa of a mouse treated with antibiotics and magnesium sulfate given *C. jejuni* (strain Cj.Vic.) by mouth. Inverted back-scatter SEM. (a) The crypts are seen to be heavily colonized with the spiral-shaped campylobacters. (b) Higher-power shot of the left boxed section in (a). (c) Higher-power shot of the right boxed section (a).

ing faster than any other organism previously observed in this type of preparation. In any one cecal scraping, four types of motility could be observed. This is stylistically represented in Fig. 7.

(i) Non-mucus associated. In areas of preparation where no tissue was present and organisms were free swimming in liquid, the motility was random, with bacteria moving in all directions.

(ii) Surface mucus associated: free movers. In some areas surface mucus could be seen heavily packed with spiral bacteria. Here the organisms moved extremely rapidly across the field of view in parallel streams. The bacteria

seem to track along the mucus strands. When the occasional organism paused or slowed down, the spiral morphology appeared particularly marked. Sometimes an individual spiral could be seen to cross at right angles to the rapidly moving stream, stop and change direction, and continue aligned to the mainstream.

(iii) Surface mucus associated: oscillating. In some areas of what was presumably surface mucus, the bacteria move but seemingly in the one spot; i.e., they move rapidly backwards and forwards, giving an oscillating effect.

(iv) Crypt mucus associated. By chance, whole crypts were sometimes preserved relatively intact in the tissue squash. The bottom and the neck of the crypt could be seen to be full of bacteria slowly moving about.

DISCUSSION

C. jejuni has been shown to colonize the intestinal mucosa of adult mice by association with intestinal mucus in both the mucus blanket and the mucus-filled crypts. No evidence of adhesion to the epithelial cells of the gut mucosa was found. This is exactly the situation found with the spiral organisms of the autochthonous microbiota that normally colonize the rodent intestinal surface. Indeed, it was similarities between these organisms and *C. jejuni* that prompted this work. Due to these similarities any model developed to study mucus colonization in the rodent by human campylobacters had to be controlled such that investigators could be certain that the organisms they were looking at were indeed the inoculated bacteria. Immunolabeling techniques at first seemed possible, but immunological cross-reactivity was found between *C. jejuni* and the majority of the natural mouse spirals and therefore could not be relied on. Use of magnesium sulfate and special combinations of antibiotics removed all spiral bacteria from the animals. The complete absence of spirals in crypts of the controls compared with the heavy colonization of the *C. jejuni*-infected animals was convincing evidence of mucus colonization, particularly considering the very rigorous "blind" nature of the assessment procedures.

These findings have considerable significance with regard to consideration of mechanisms of pathogenicity of this human gut pathogen. However a pathogenic bacterium finally causes tissue damage or produces symptoms in the host, it must have evolved a way of associating with the intestinal mucosa either as the first stage or an invasive process or to get close enough to tissue cells for irritant or toxic products to act. Understanding the basis of this first stage of pathogenesis is important as it is likely to provide a basis for a practical prevention of intestinal disease.

Freter (4) comments that it is self-evident that active or passive penetration of mucus gel will have a similar ecological function as binding to the epithelial cell surfaces. He also suggests that in some instances trapping in the mucus gel may even represent the only way in which bacteria associate with the mucosa. The observations in the mouse cecal model support this view, and yet at the moment adhesion to tissue is being consistently put forward as a possible determinant of pathogenicity for *C. jejuni*. Thus, in one of the more recent reviews on this topic, Skirrow (18) concludes that an ability to adhere to gut epithelium is an important factor, quoting a number of papers on mouse colonization.

Close examination of the results in references cited in this review show that the case for adhesion is "not proven." Thus, scanning micrographs presented by Field et al. of *C. jejuni* on infant mouse gut are consistent with colonization of the mucus (3). Similarly, the large mats of campylobacters on the mucosal surface of the colons of mice seen by Merrell et al. (7) within 48 h of intraileal inoculation are likely to be mucus associated. Experiments of Newell and Pearson (9) with in vitro preparations of human epithelial cell lines suggest that flagella might play a role in attachment, although this was only observed after impaction of organisms onto cells by centrifugation, a highly artificial system, hardly suggestive of a firm bacterium-cell surface association. SEM is an inappropriate technique for the demonstration of adhesion as it provides no information on the bacterium-cell surface interface and there are potentially many fixation artefacts. Workers in the field of adhesion rely much more on TEM (1). A transmission photograph in the Newell paper of the intestinal mucosa of an infant mouse colonized with *C.*

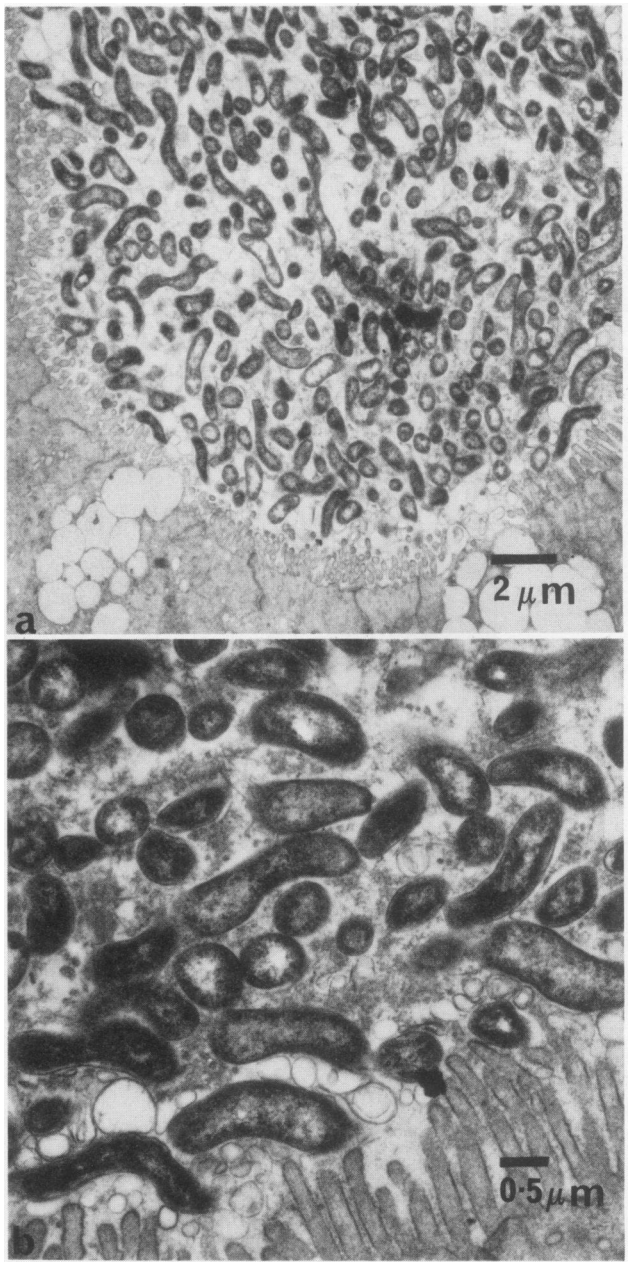

FIG. 4. Bottom of a crypt of a *C. jejuni*-colonized mouse showing dense packing of bacteria and close proximity to the tissue surface. (a) TEM; (b) TEM.

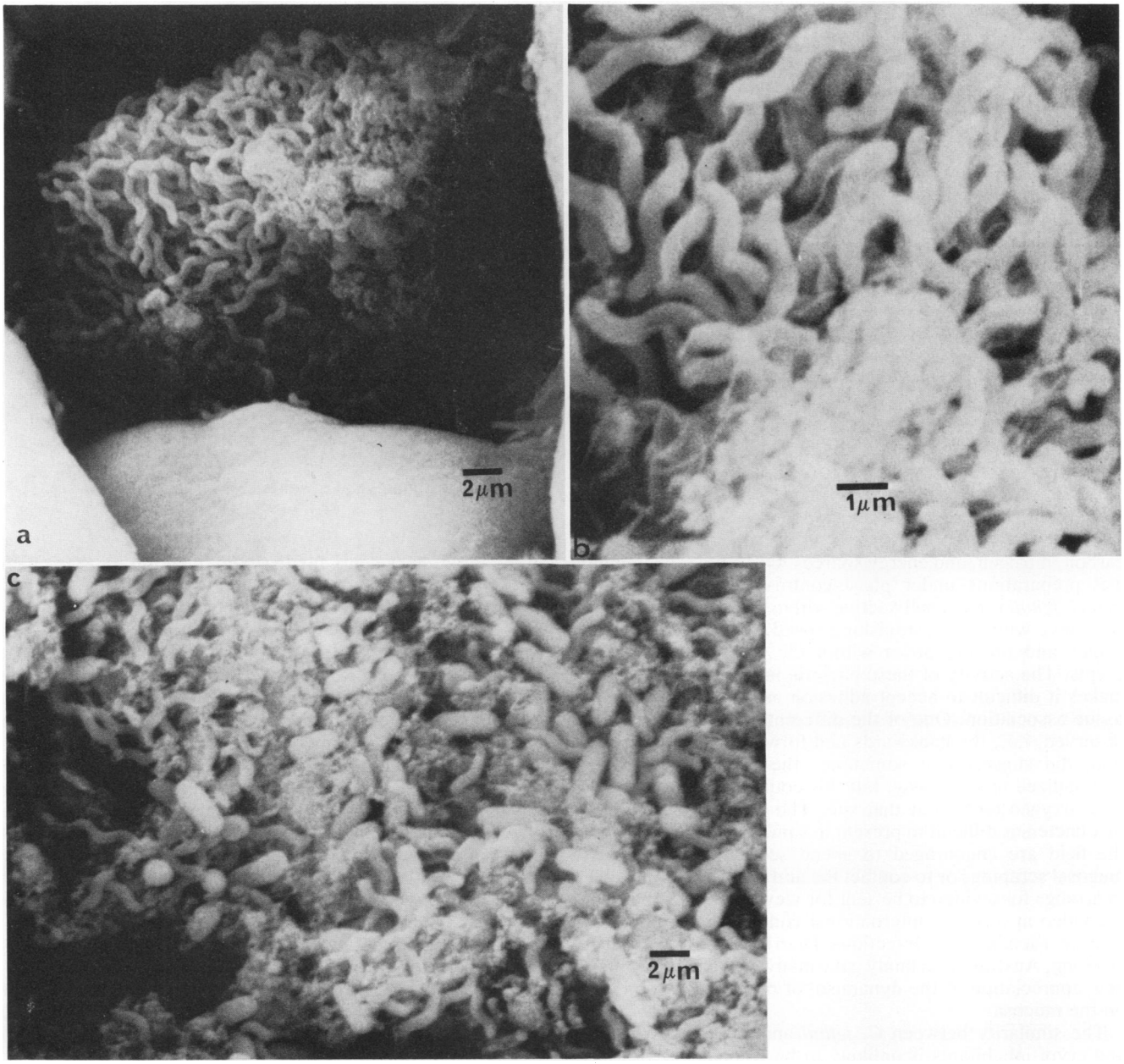

FIG. 5. Cecal epithelium of a *C. jejuni*-colonized mouse. (a) Opening of a crypt (SEM); (b) same crypt as in (a) (SEM); (c) surface mucus showing spiral- and rodshaped bacteria (SEM).

jejuni is cited as evidence of attachment to the intestinal epithelial microvilli but only shows crypt colonization.

The reason for this preoccupation of many workers with adhesion is probably twofold. First, statements such as "All enteric pathogens must attach" (3) are now well entrenched not only in the research literature on intestinal disease but also in texts on microbiology (19). Thus, whenever a new pathogen is discovered, there is a flurry of activity to look for specific adhesins without sufficient attention to whether there is evidence for adhesion to intestinal surfaces. Often the assessment procedures involve adhesion to mammalian cells in vitro without comparative control studies with nonpathogens. Second, the static image presented by photomicrographs and difficulty in adequately preserving intestinal mucus encourages a perception of adhesion, particularly when investigators are looking for it. Certainly Fig. 6, taken of the cecal mucosa of one of our colonized animals, could have been presented as evidence of adhesion. Only after long examination of preparations with a wide variety of microscopic methods, particularly observation of living wet preparations, could the importance of mucus colonization be clearly appreciated.

Savage (14) also stated that a reasonable theoretical case could be made that microbes can colonize mucus on a particular surface and only appear in microscopic preparations to adhere to epithelial cells underlying the mucus. He suggested that they may be able to move about in the mucus layer and even digest its constituents and use them as

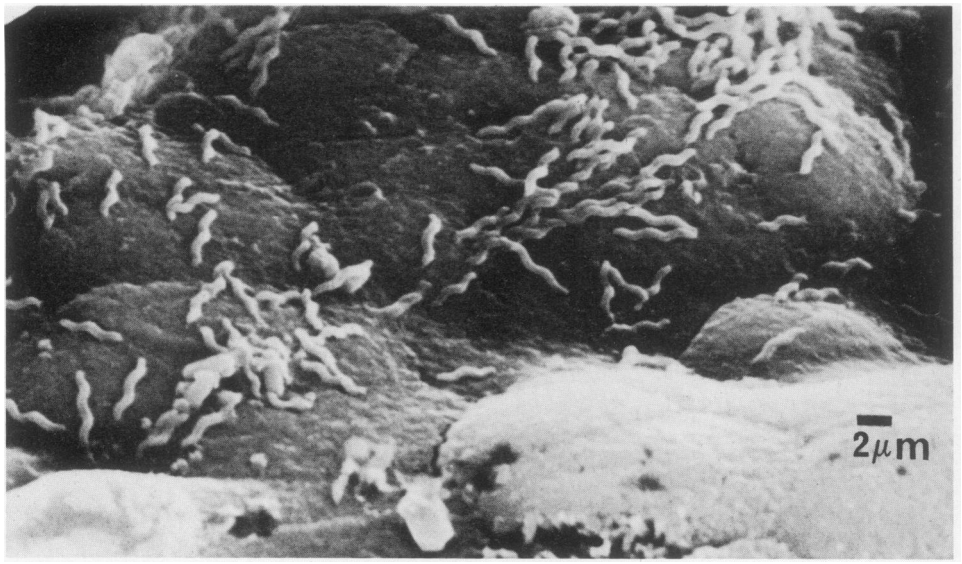

FIG. 6. *C. jejuni* on the cecal epithelium of a colonized mouse giving the impression of adhesion to tissue (SEM).

carbon, nitrogen, and energy sources. Our studies examining wet preparations under phase-contrast microscopy show that *C. jejuni* is extremely active within intestinal mucus and can move with quite astonishing speed up and down mucus stands and up and down within the mucus in the cecal crypts. The activity of these bacteria in the intestinal tissue makes it difficult to accept adhesion as a major method of tissue association. One of the different types of movement observed, i.e., the backwards and forwards oscillatory motion, did suggest that sometimes the organism could be immobilized in some way, but this could also be due to the local oxygen tension at that site. The motility of the campylobacters is difficult to present in a publication. Workers in the field are encouraged to spend some time looking at mucosal scrapings or to contact the authors who may be able to arrange for a video to be sent for viewing. Presentation of the video at a recent international conference on diarrheal disease (Seminar on Infectious Diarrhoea in the Young, Geelong, Australia) certainly gave many of the participants a new appreciation of the dynamism of organisms within and on the mucosa.

The similarity between *C. jejuni* and the normal mucus and crypt inhabitants is unlikely to be coincidence. As has been discussed before, a spiral morphology and special mode of motility are likely to give these organisms a selective advantage in the viscous mucus environment (6). Unpublished experiments have shown that the intestinal spirals and *C. jejuni* will move more efficiently in solutions of high viscosity compared with motile rod-shaped intestinal bacteria. One member of the normal rat microbiota which has been studied in pure culture in colonization studies has a very characteristic spiral morphology and can be easily recognized in histological studies (10). This organism has been shown to colonize the cecal crypts in animals in exactly the same way as *C. jejuni*. Significantly, these bacteria not only share a spiral morphology but also the same gaseous growth requirements; i.e., they are microaerophiles with a requirement for carbon dioxide. As the cecal contents are known to be highly reduced (5), it is not surprising that the microaerophiles congregate close to the oxygenated tissue surface. Thus, both spiral morphology and microaerophilism are equal requirements for tissue association by these bacteria.

To be satisfied that mucus colonization alone is an adequate means of association with intestinal epithelium and that adhesion need not be postulated, one must be sure that organisms can achieve a high enough quantity and a close enough proximity to tissue to exert their final pathogenic potential whatever that may be. The figures shown above and examination of many other tissue sections have convinced us that these two criteria are fulfilled in the mouse cecal model. If *C. jejuni* produced either enterotoxin or tissue irritant products in these animals, they are close enough to have an effect. The reason for lack of pathological changes must therefore be due to the mouse tissue being refractory to the bacterial products or that the synthesis of these products has not been switched on or even because they are unable to adhere to mouse surfaces.

In another animal model where crypt colonization does appear to have occurred, a pathology is observed. Inflammatory bowel disease has been studied in a colony of marmosets; destruction of crypts and crypt abscesses were found to be associated with colonization by *C. jejuni* (R. L. Cisneros, A. B. Onderdonk, R. Bronson, and P. Shegal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B57, p. 24). An interesting, but neglected paper published in 1974 describes lesions in weaning rabbits with diarrheal disease of unknown cause. Crypt lesions were an important feature of this disease and crypts were seen to be full of what looks very much like *C. jejuni* (8). Histologic sections of human biopsies from patients with severe infection show acute colitis with inflammatory infiltration of the lamina propria and crypt abscesses as important features (12). Closer examination of these specimens is required to demonstrate crypt colonization.

An important consideration of this work is the relevance of the mouse cecal model to a study of human campylobacteriosis. As has been mentioned, many of the currently accepted views on the pathogenesis of *C. jejuni* infection are based on studies in rodents (18) so the precedent is there. However, much of the evidence for mucus colonization in humans is anecdotal; for example, spiral organisms are seen

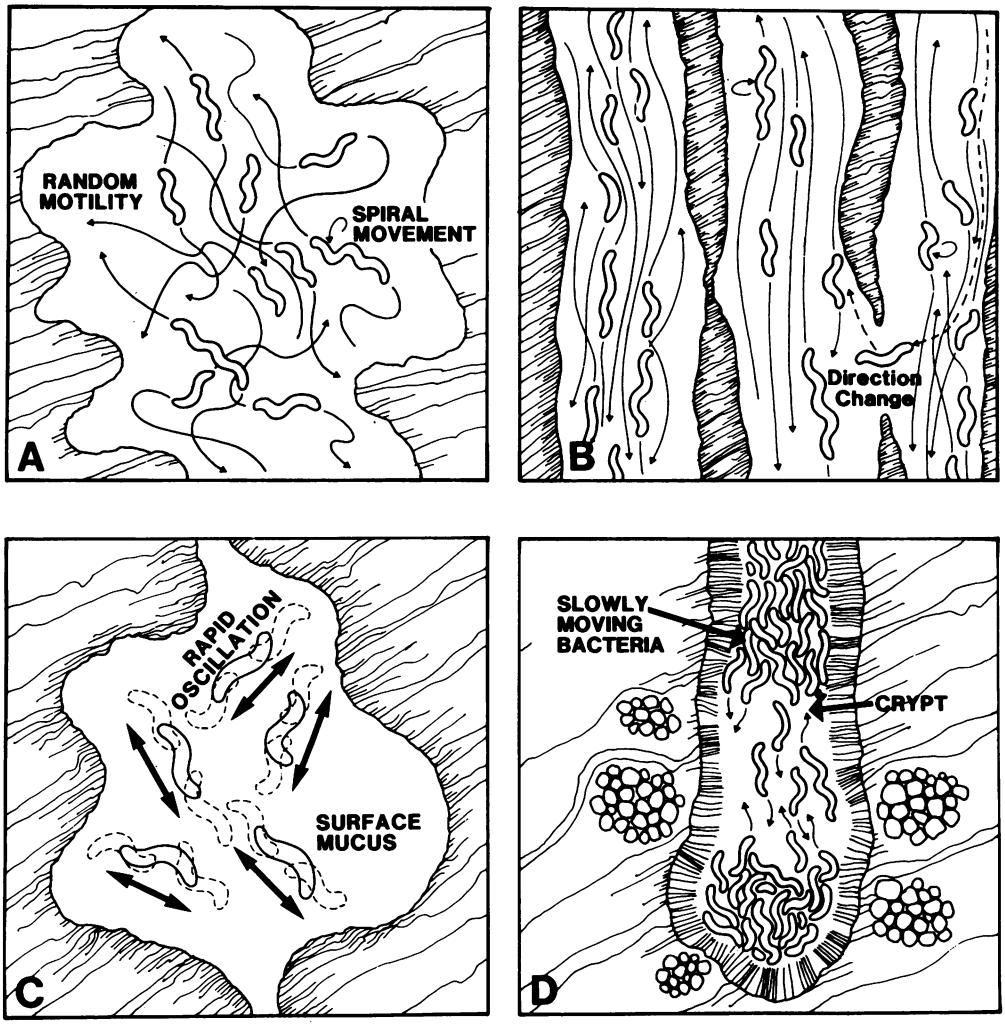

FIG. 7. Different types of movement of *C. jejuni* observed in fresh wet scrapings of cecal epithelium of colonized mice. (A) Non-mucus associated; (B) surface mucus associated: free movers; (C) surface mucus associated: oscillating; (D) crypt mucus associated. (Graphics by Richard Jones, Audiovisual Unit, University of New South Wales.)

to be associated with mucus flecks in the stools of patients with campylobacter diarrhea. There have been no studies on human infection that have used techniques that preserve intestinal mucus. Based on the observations above there is a need for such studies.

However, it is likely that the colonization of rodent intestinal mucus is relevant to the pathogenesis of human infection. We consider the analogy of the natural mechanisms of mucosal association as discussed in the introduction to be very strong, and the possibility of pathogens being able to associate with epithelia by adaptation to the gut mucus is highly probable, with *C. jejuni* as a likely candidate. If this is so, then much of the research effort looking for specific adhesins in campylobacter infection may be misdirected. A major determinant of pathogenicity in intestinal infection with *C. jejuni* is proposed to be an ability to colonize intestinal mucus. Better understanding of the mechanism of this association and the properties of the bacterium that are responsible will provide a basis for the rational selection of preventative measures, just as studies on gut pathogens known to associate with tissue via specific receptors has provided a rational basis, e.g., *Escherichia coli* vaccines. The model of mucus association in adult antibiotic-treated mice described above provides an opportunity for colonization studies with variant organisms and immunization studies.

ACKNOWLEDGMENTS

This work was supported by The National Health and Medical Research Council of Australia, The Medical Research Council of Canada, and The Natural Science and Engineering Council of Canada.

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