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Antigenic Analysis of *Campylobacter* Species and an Intracellular *Campylobacter*-Like Organism Associated with Porcine Proliferative Enteropathies

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Whole-cell and outer membrane preparations of *Campylobacter mucosalis*, *C. hyointestinalis*, *C. jejuni*, and *C. coli* isolated from porcine intestines were compared with preparations of intracellular *Campylobacter*-like organisms extracted directly from the lesions of pigs with proliferative enteropathy. By gradient polyacrylamide gel electrophoresis, outer membrane and total protein profiles of *C. mucosalis*, *C. hyointestinalis*, *C. jejuni*, and *C. coli* were significantly different from each other and from those of the *Campylobacter*-like organisms. Immunoblotting of these preparations with rabbit antisera or monoclonal antibodies prepared against the intracellular *Campylobacter*-like organisms showed strong reactions only with a 25,000- to 27,000-molecular-weight component of preparations of the intracellular organisms. Antisera to cultivable *Campylobacter* species isolates did not react with preparations of intracellular organisms. Isoelectric focusing of sonicated preparations showed protein profile differences and an immune-reactive component in the intracellular organisms with a pI of 4.5. This study suggests that the intracellular *Campylobacter*-like organism associated with proliferative enteropathy may be a novel bacterium with significant antigenic differences from the *Campylobacter* species previously associated with the disease.

Proliferative enteropathy, or enteritis, is a major disease of weaned pigs worldwide, clinically manifested by poor weight gain (25). Pathologically it is characterized by adenomatous hyperplasia of immature ileal enterocytes containing intracytoplasmic, *Campylobacter*-like organisms (24). This lesion, often known as intestinal adenomatosis, may be complicated by mucosal necrosis or intraluminal hemorrhage, conditions known as necrotic enteritis and hemorrhagic enteropathy, respectively (25). The consistent presence in all these lesions of intracellular *Campylobacter*-like organisms suggests that they are the etiological agent (25, 26). Bacteriologic culture of the lesions frequently yields *Campylobacter muscosalis* or *C. hyointestinalis*, and neither of these organisms is numerous in normal porcine intestines (7, 12, 21). *C. jejuni* and *C. coli* have also been isolated from the lesions; however, these bacteria are considered part of the normal flora (23, 26). Oral dosing of conventional or gnotobiotic piglets with any of these cultured *Campylobacter* species does not cause any significant disease (3, 8, 17).

Proliferative enteropathy has been reproduced only by orally dosing pigs with homogenates of naturally affected mucosa (16, 22). No particular *Campylobacter* sp. was clearly associated with reproduction of the disease in those experiments. Therefore, experimental and cultural results have not identified the intracellular organism as one of the *Campylobacter* species that can be grown from porcine intestines.

Furthermore, rabbit antisera prepared against whole cells of each *Campylobacter* sp. mentioned did not react with intracellular organisms in indirect immunofluorescence assays (13). In the same study, antisera prepared against intracellular *Campylobacter*-like organisms purified from lesions by selective filtration did react with intracellular bacteria in sections of lesions from other pigs. These results showed specific immunological differences between the reactivity of culturable *Campylobacter* species and that of the intracellular organisms in proliferative enteritis. This difference may reflect other major differences or merely the presence of an antigen uniquely reactive in the intracellular location.

The objectives of this study were to specify antigens of the intracellular organisms and to compare them with those of *Campylobacter* species associated with the disease and found in porcine intestines. We previously reported some preliminary results for our preparation of monoclonal antibodies to the intracellular organisms (18).

MATERIALS AND METHODS

***Campylobacter* strains.** The strains of *C. mucosalis*, *C. hyointestinalis*, *C. jejuni*, and *C. coli* used in this study are detailed in Table 1. All strains were cultured on Columbia-base agar (Oxoid Ltd., London, England) supplemented with 5% sheep blood. Growth conditions were 5% O_2, 10% CO_2, and 70% H_2 at 37°C for 48 h. Antigens from whole cells of each strain were prepared in Tris buffer, pH 8.0, by sonication (M.S.E. Instruments). Outer membranes were prepared from selected strains (Table 1) by the EDTA-lysozyme method of Mills and Bradbury (19). The mean protein concentration in each preparation was 0.3 ± 0.05 μg ml^{-1}. *C. mucosalis* 1248/72 and *C. hyointestinalis* 124/73 A4 flagellins were also prepared by the method of Logan and Trust (15).

Porcine intestinal material. Intracellular *Campylobacter*-like organisms were directly extracted from the intestines of three pigs (Table 2) affected by proliferative enteritis. Two of these intestines were selected because cultivable *Campylobacter* spp. were present in small numbers (Table 2). Intracellular bacteria were extracted by a modification of the

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TABLE 1. *Campylobacter* spp. strains studied

Species	Strain	NCTC[a] designation	Origin	Pathology at necropsy
C. mucosalis	1248/72[b]	11000	Porcine ileum	Intestinal adenomatosis
	124/73B4		Porcine ileum	Necrotic enteritis
C. hyointestinalis	124/73A4[b]	11562	Porcine ileum	Necrotic enteritis
	9AL3		Porcine ileum	Proliferative enteritis
C. jejuni[c]	1268/84J[b]		Porcine ileum	Proliferative hemorrhagic enteropathy
	664/83		Hamster ileum	Normal
C. coli	9BF2		Porcine feces	Proliferative enteritis
	9AF3a		Porcine feces	Proliferative enteritis

[a] NCTC, National Collection of Type Cultures, Colindale, London.
[b] Outer membrane preparations made.
[c] Antisera were not prepared against *C. jejuni* 1268/84J.

method of Lawson et al. (13). Briefly, portions of each mucosa were washed in phosphate-buffered saline (pH 7.4), cells were dispersed by mixing the mucosa in 0.75% trypsin in phosphate-buffered saline for 40 min at 37°C, and intracellular bacteria were released by homogenization in a blender. After clarification by centrifugation, the resulting suspensions were passed through filters (2.5-, 1.0-, and 0.8-μm pore size; Millipore Corp., Bedford, Mass.). Smears of these suspensions showed numerous bacteria, almost exclusively of *Campylobacter* morphology, with little background material. Mucosa from a normal pig (Table 2; sample 204/79) was washed and homogenized. Mucosal bacteria and control preparations were then separately sonicated and adjusted for protein concentration to 0.3 μg ml^{-1}. Examination of cultures for *Campylobacter* species was carried out with selective media and dilution methods by using standard techniques.

Rabbit sera. Antisera to the strains listed in Table 1 and to mucosal bacteria (mucosa 1269/76) were raised by injecting rabbits intravenously with 0.5 ml of Formalin-fixed whole-cell antigen at intervals and concentrations described previously (12). Antiserum to an unrelated organism, "*Haemophilus somnus*" 578/77, was prepared identically as a negative control.

Mouse immunization. Intracellular *Campylobacter*-like organisms from mucosa 284/86 (Table 2) were further purified by passage of the suspension through a wheat germ agglutinin-agarose column (Sigma Chemical Co., St. Louis, Mo.). Preliminary study had established that wheat germ agglutinin bound to *C. coli* and cellular debris isolated from this porcine mucosa. Such binding has been reported previously for other *C. coli* strains and intestinal cell material (5, 30). Formalin-fixed whole-cell bacterial suspensions were prepared for use as immunogen from the unbound fraction of the column and from the fraction eluted from the column with 0.2 M *N*-acetyl-D-glucosamine. Adult female BALB/c mice were injected intramuscularly with 0.3 ml of each suspension (approximately 0.3 μg of protein ml^{-1}) on 0.3 ml of Freund complete adjuvant and then four times intravenously with 0.2 ml of suspension at 12-week intervals. Serum was collected from each mouse and screened by immunofluorescence and the enzyme-linked immunosorbent assay, initially for reactivity to intracellular *Campylobacter*-like organisms and secondarily for whole-cell antigens of each of the *Campylobacter* spp. strains (Table 1); conditions used for these assays have been described elsewhere (13, 28).

TABLE 2. Porcine intestinal material used and organisms in smears thereof

Sample no.	Pathology[a]	*Campylobacter*-like organisms in mucosal smears[b]	*Campylobacter* sp. cultured[c] (estimated no. of organisms)
1269/76	PHE	+	*C. coli* (2.0)
284/86	PHE	+	*C. coli* (4.5)
761/86	PIA	+	*C. mucosalis* (6.4) *C. coli* (5.2)
204/79	None	−	None

[a] PHE, Proliferative hemorrhagic enteropathy; PIA, porcine intestinal adenomatosis.
[b] +, >10 *Campylobacter*-like organisms per high-power field (modified acid-fast stain); −, no organisms detected.
[c] Mucosae (except mucosa 204/79) were filtered in phosphate-buffered saline; 1 ml of 0.8-μm filtrate was cultured. Data are expressed as $\log_{10}$ per milliliter.

Monoclonal antibody production. The mice were killed 4 days after the last injection. Spleen cells from immunized animals were hybridized with NSO mouse myeloma cells (6). Culture supernatant fluids were screened as the mouse sera were. Hybridomas reactive with intracellular *Campylobacter*-like organisms but not reactive with *Campylobacter* species were cloned twice in soft agar. After cloning, the hybridoma cell lines were expanded in culture for further testing. Antibody isotype was determined by a commercial gel diffusion kit (Serotec).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sonicated preparations or outer membranes were mixed with a reducing buffer containing 5% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, 2% (wt/vol) sodium dodecyl sulfate, and 0.025 M Tris base. Samples were boiled at 100°C for 3 min, and 5 μg of protein was applied to each lane. Vertical slab gel electrophoresis was performed by the method of Laemmli (11) by concentrating proteins in a 4.5% stacking gel and separating them in a 7 to 20% gradient gel. Electrophoresis was continued at 80 V for 18 h.

Isoelectric focusing. Isoelectric focusing was performed in 1-mm-thick, 5% polyacrylamide gels containing ampholytes (pH 4 to 8) on a flat-bed apparatus with a Teflon-coated aluminum top and cooling equipment (Pharmacia, Uppsala, Sweden). After the gel was prefocused for 500 V-h, sonicated preparations were loaded onto the gel and electrofocusing continued at a constant power setting of 30 W for 4,000 V-h (1).

Immunoblotting. Proteins from each gel were transferred onto nitrocellulose papers with a semidry graphite electrode apparatus (Ancos, Denmark). Two sheets of Whatman 3MM filter paper were soaked in a transfer buffer (0.039 M glycine, 0.048 M Tris buffer, 0.0375% [wt/vol] sodium dodecyl sulfate, 20% [vol/vol] methanol) and placed on the anode. The nitrocellulose paper, the gel, and two additional sheets of soaked filter paper were placed on top, and then the cathode

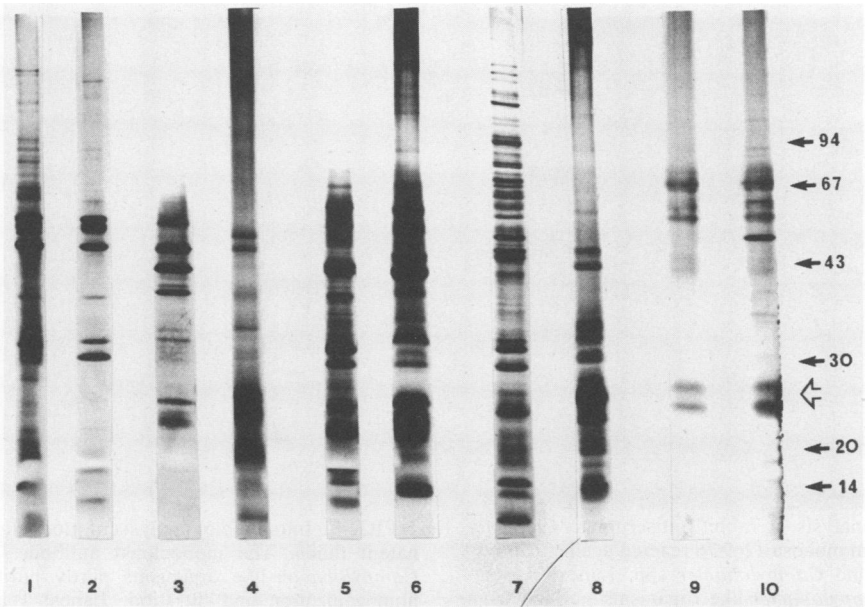

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis preparations of *Campylobacter* spp. and purified *Campylobacter*-like organisms. Lanes: 1, outer membrane preparation of *C. mucosalis* 1248/72; 2, outer membrane preparation of *C. mucosalis* 124/73B4; 3, outer membrane preparation of *C. hyointestinalis* 124/73A4; 4, sonicated preparation of *C. hyointestinalis* 9AL3; 5, outer membrane preparation of *C. jejuni* 1268/84J; 6, sonicated preparation of *C. jejuni* 664/83; 7, sonicated preparation of *C. coli* 9BF2; 8, sonicated preparation of *C. coli* 9AF3a; 9, whole-cell preparation of *Campylobacter*-like organisms purified from mucosa 284/86 by homogenization, filtration, and passage through a wheat germ agglutinin-agarose column; 10, sonicated preparation of *Campylobacter*-like organisms purified from mucosa 284/86 as described for lane 9. Silver stain; molecular weights are expressed in thousands. The open arrow indicates the 25K and 27K bands.

was attached. Electroblotting was carried out at a constant voltage of 24 V for 90 min. Unreacting protein-binding sites in the nitrocellulose paper were blocked by incubation for 4 h with Tris buffer containing gelatin and 4% (vol/vol) goat serum. Appropriate antisera at a dilution of 1:200 or neat hybridoma supernatant fluid was reacted with the blots for 16 h. The blots were then washed and probed with peroxidase-conjugated antiserum to immunoglobulins of the appropriate species. After being washed, antigen bands were visualized with a development solution of 0.02% (wt/vol) 4-chloro-naphthol in Tris buffer containing 0.001% (vol/vol) hydrogen peroxide.

RESULTS

Polyacrylamide gel electrophoresis. The protein profiles of *Campylobacter* spp. and the product of *Campylobacter*-like organisms from mucosa 284/86 obtained after passage through a wheat germ agglutinin-agarose column are shown in Fig. 1. The protein profile obtained with each *Campylobacter* sp. was distinct. Sonicated whole-cell preparations were identical to outer membrane preparations from the same strain, except for minor differences in protein bands. The profiles for *C. jejuni* and *C. coli* strains were dominated by two major outer membrane protein bands of 40,000 and 45,000 molecular weight (40K and 45K proteins), together with six to eight other prominent protein bands. The profiles of *C. mucosalis* strains were dominated by a triplet of major outer membrane protein bands with molecular weights between 50,000 and 65,000 and either four or five other prominent proteins. The *C. hyointestinalis* strain profiles had 45K and 50K major outer membrane protein bands, with either three or four other prominent proteins.

The protein profiles of the *Campylobacter*-like organisms extracted from porcine proliferative enteropathy tissue were dominated by major protein bands of 55,000 and 70,000 molecular weight. Minor components were recognized between 20,000 and 43,000, including two distinct bands at 25,000 and 27,000. The normal tissue sample (mucosa 204/79) had minor distinct components of 23,000 and 60,000.

Immunoblotting and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Antisera to *Campylobacter*-like organisms from mucosa 1269/76 recognized only the 25K and 27K components of *Campylobacter*-like organisms from mucosae 284/86 (Fig. 2), 1269/76, and 761/86. There was also recognition of the 55K and 43K protein bands in some preparations of mucosa 284/86 (Fig. 2, lane 2). This serum showed only a slight reaction with the major outer membrane protein band of *C. jejuni* and did not react with any of the other *Campylobacter* species tested or with normal tissue (mucosa 204/79).

Antibodies in the *C. jejuni* and *C. coli* antiserum recognized the major outer membrane proteins of homologous and other strains of *C. jejuni* and *C. coli*. Several previous reports contain illustrations of these reactions (2, 14, 15).

Antibodies in antisera to *C. mucosalis* 1248/72 and 124/73B4 recognized the outer membrane proteins of the *C. mucosalis* strains tested. These antisera also recognized a 60K protein band in lanes containing the flagellin preparation of *C. mucosalis* 1248/72, and minor reactions were evident with proteins of other *Campylobacter* spp. Similarly, antisera to *C. hyointestinalis* 124/73A4 and 9AL3 recognized major outer membrane protein bands of *C. hyointestinalis*. These antisera also recognized some outer membrane proteins of *C. mucosalis* and *C. jejuni* strains.

No further reactions between any antiserum to *Campylobacter* species and any mucosal preparation were evident. Rabbit antiserum 578/77 did not recognize any antigens tested.

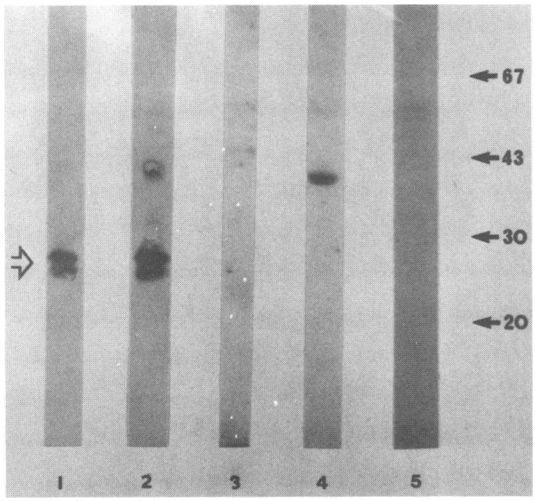

FIG. 2. Immunoblot analysis of rabbit antiserum to *Campylobacter*-like organisms from mucosa 1269/76 reacted against *Campylobacter*-like organisms and *Campylobacter* spp. Lanes: 1, sonicated preparation of *Campylobacter*-like organisms purified from mucosa 284/86 by homogenization, filtration, and passage through a wheat germ agglutinin-agarose column; 2, sonicated preparation of *Campylobacter*-like organisms partly purified from mucosa 284/86 by homogenization and filtration; 3, sonicated preparation of *C. mucosalis* 1248/72; 4, outer membrane preparation of *C. jejuni* 1268/84J; 5, sonicated preparation of *C. coli* 9BF2. Molecular weights are expressed in thousands. The open arrow indicates the 25K and 27K bands.

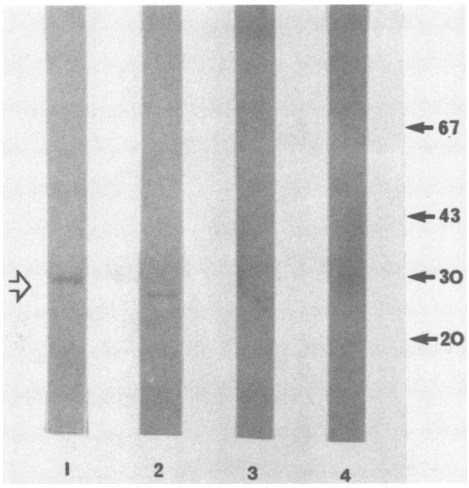

FIG. 3. Immunoblot analysis of monoclonal antibodies in supernatant fluids. The monoclonal antibodies were reacted against *Campylobacter*-like organisms partly purified from mucosae by homogenization and filtration. Lanes: 1 and 2, mucosa 284/86, antibodies 4F5 and IG4, respectively; 3, mucosa 1269/76, antibody IG4; 4, mucosa 761/86. Molecular weights are expressed in thousands. The open arrow indicates the 25K and 27K bands.

Characterization of monoclonal antibodies. Two monoclonal antibodies, IG4 and 4F5, reacted only with intracellular *Campylobacter*-like organism preparations and not with normal pig material or cultured *Campylobacter* species. Isotyping indicated that these antibodies were immunoglobulin G3. Typical immunofluorescence reactions showed brightly fluorescing curved bacilli at supernatant fluid dilutions up to 1:10. The enzyme-linked immunosorbent assay gave positive color reactions (optical density, >0.2) at supernatant fluid dilutions up to 1:1,000.

Antibodies in these supernatant fluids recognized the 25K and 27K protein bands of *Campylobacter*-like organisms from mucosae 284/86, 1269/76, and 761/86 (Fig. 3) in immunoblots. No reaction between these antibodies and cultured *Campylobacter* species antigen or normal mucosa (sample 204/79) was observed.

Isoelectric focusing and immunoblotting. The focused protein profiles of sonicated whole-cell antigens of selected *Campylobacter* species, as well as the products of *Campylobacter*-like organisms from mucosae 284/86 and 761/86 after passage through separate wheat germ agglutinin-agarose columns, are shown in Fig. 4. The profiles of the *Campylobacter*-like organisms show prominent protein bands with isoelectric points (pIs) between 6 and 7, with much material still adjacent to the sample application sites at pI 4.5 and two prominent proteins with pIs close to 5.0, particularly in the preparation of mucosa 284/86.

The profiles for *C. mucosalis* strains show a major protein band with a pI of 6.5, particularly in the mucosa 1248/72 sample. The profiles for *C. hyointestinalis*, *C. jejuni*, and *C. coli* strains show two proteins around pI 6.0, at slightly differing sites. In all the *Campylobacter* spp., minor protein bands are evident between pIs 7 and 8.

Antiserum to *Campylobacter*-like organisms from mucosa 1269/76 and in supernatant fluids IG4 or 4F5 recognized only the pI 4.5 protein bands of organisms from mucosa 284/86 (Fig. 4) and mucosa 761/86. These antibodies did not recognize components from any of the *Campylobacter* species tested.

DISCUSSION

Intracellular *Campylobacter*-like organisms were purified from lesions of pigs with proliferative enteritis and com-

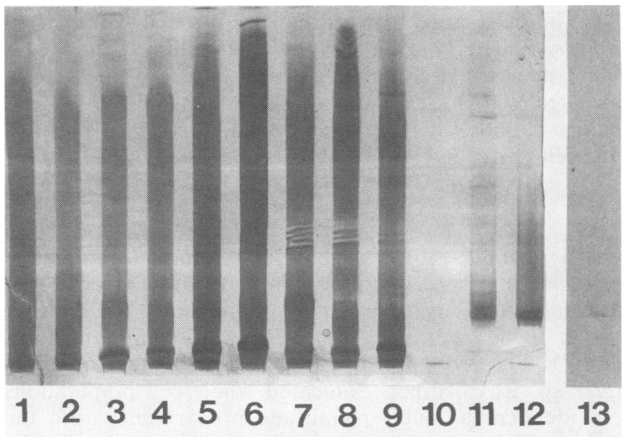

FIG. 4. Isoelectric-focusing gel analysis (lanes 1 to 12; silver stain) and immunoblot analysis (lane 13) of preparations of *Campylobacter*-like organisms. Lanes: 1, sonicated preparation of *C. mucosalis* 1248/72; 2, sonicated preparation of *C. mucosalis* 124/73B4; 3, sonicated preparation of *C. hyointestinalis* 124/73A4; 4, sonicated preparation of *C. hyointestinalis* 9AL3; 5, sonicated preparation of *C. jejuni* 1268/84J; 6, sonicated preparation of *C. jejuni* 664/83; 7, sonicated preparation of *C. coli* 9AF3a; 9, sonicated preparation of *C. coli* NCTC 11353; 10, isoelectric marker, pI 4.0; 11, sonicated preparation of *Campylobacter*-like organisms from mucosa 284/86; 12, sonicated preparation of *Campylobacter*-like organisms from mucosa 761/86; 13, monoclonal antibodies in supernatant fluid IG4, reacted against *Campylobacter*-like organisms from mucosa 284/86.

pared, by means of gel electrophoresis protein profiles and immunoblotting reactions, with *Campylobacter* species culturally associated with the disease. The intracellular organisms tested had a distinctive protein profile, and 25K and 27K proteins reacted specifically with antibodies in rabbit antisera and with mouse monoclonal antibodies raised against the intracellular organisms. This suggested that the intracellular organisms had a distinctive antigenic profile and that their 25K and 27K components were the major antigenic components. These components were not detected in known *Campylobacter* spp. or in normal porcine intestine. Thus the intracellular organism may be new, uncultured bacterium or may be one or more of the known *Campylobacter* spp. which undergo a marked change in the molecular sizes and antigenicities of their outer membrane components during intracellular growth.

Campylobacter spp. have been shown to have different outer membrane protein profiles in gel electrophoresis, thus reflecting valid genetic differences among species; except that *C. jejuni* and *C. coli*, which have nearly 30% DNA homology, have similar profiles (2, 14, 29). Therefore, the different profile of the purified *Campylobacter*-like organisms indicates that they are not related to any of the *Campylobacter* spp. tested. The use of either sonication or outer membrane preparation methods has previously been shown not to significantly alter *Campylobacter* sp. profiles (2). Flagellin is a highly conserved, 60K component of *Campylobacter* spp. (15, 29). It is also a dominant immunogen and can cross-react with antibodies prepared against heterologous *Campylobacter* sp. flagellin (15, 29). It was detected in those cultured *Campylobacter* spp. tested here, but no 60K component was recognized in the intracellular *Campylobacter*-like organisms.

Monoclonal antibodies specific to the intracellular *Campylobacter*-like organisms in proliferative enteritis lesions were produced. Immunoblotting results suggested that these antibodies bound to the 25K to 27K outer membrane component present only in the intracellular organisms. Reactions with this component could not be detected in assays with normal pig intestine or *Campylobacter* sp. antigen. This confirms the uniqueness of this component to the *Campylobacter*-like organisms, a result which could not be ascertained with confidence when polyclonal rabbit antisera were used, because of some reactions with a variety of *Campylobacter* sp. antigens.

Specific antibodies were produced only after the mice were immunized with affinity-purified antigen. The use of whole bacteria carefully purified from cellular material has also been successful in producing specific monoclonal antibodies with nonculturable bacteria, such as *Mycobacterium leprae*, purified from tissues (9).

Intracellular *Campylobacter*-like organisms in lesions of hamsters with proliferative enteritis have been reacted previously with monoclonal antibodies to *C. jejuni* with negative results, suggesting that the intracellular organisms were distinct from recognized *Campylobacter* spp. (27). However, that study did not include antibodies specific to the intracellular organisms. Previous studies of monoclonal antibodies prepared against *C. jejuni*, *C. coli*, *C. fetus*, or *C. pyloridis* have confirmed the ability of the antibodies to distinguish *Campylobacter* species from one another and to bind to various components of the outer membranes of the organisms (4, 10).

Isoelectric focusing results suggested that *Campylobacter*-like organisms in proliferative enteritis lesions possess a specific component of pI 4.5 with an antigenic site identical to that of the 25K to 27K component detected in preparations in reducing gels. Therefore, it is likely that the components detected by the two methods represent the same structural component. Isoelectric focusing is a nondenaturing method, indicating that there is one major antigen and that the sodium dodecyl sulfate-polyacrylamide gel electrophoretic procedure did not denature this or other significant compounds. The absence of components in other *Campylobacter* spp. shown to be reactive with monoclonal antibodies by isoelectric focusing further suggests that these organisms are antigenically different from known *Campylobacter* species.

The presence of protein bands of 25,000 to 27,000 in preparations of the intracellular organism suggests a minor change in molecular structure, possibly caused by the addition of glycosyl groups, in one basic component. Similar paired protein bands of 60,000 to 62,000 have been recognized in reactions of monoclonal antibodies to *C. jejuni* (20). Immunofluorescence results suggest that the 25K to 27K proteins are major components of the outer membrane of the intracellular organisms. No evidence of this component being pig derived was detected.

This study suggests that the intracellular *Campylobacter*-like organism(s) is a novel bacterium not yet cultured by conventional methods and that it may be the etiological agent of the proliferative enteropathies.

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