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Immunocytochemical Evidence of *Listeria*, *Escherichia coli*, and *Streptococcus* Antigens in Crohn’s Disease

YING LIU,* HERBERT J. VAN KRUININGEN,* A. BRIAN WEST,* RICHARD W. CARTUN,§ ANTOINE CORTOT, II and JEAN-FRÉDÉRIC COLOMBEL II

*Department of Pathobiology, University of Connecticut, Storrs, Connecticut; †Department of Pathology, Yale University, New Haven, Connecticut; §Department of Pathology, Hartford Hospital, Hartford, Connecticut; and IIRegistre des Maladies Inflammatoires du Tube Digestif du Nord-Ouest de la France, Centre Hospitalier Régional et Universitaire de Lille, Lille, France

See editorial on page 1589.

Background/Aims: Infectious agents have long been suspected of playing a role in the initiation of Crohn’s disease. The objective of this study was to search for likely microbial agents in diseased tissues using immunocytochemical techniques. Methods: Intestines and mesenteric lymph node specimens of 21 patients from two French families with a high frequency of Crohn’s disease and from Connecticut were studied. The microbial agents searched for included *Bacteroides vulgatus*, *Borrelia burgdorferi*, *Escherichia coli*, *Listeria monocytogenes*, *Streptococcus* spp., bovine viral diarrhea virus, influenza A virus, measles virus, parainfluenza virus, and respiratory syncytial virus. Results: Seventy-five percent of the patients with Crohn’s disease (12 of 16) were positively labeled with the antibody to *Listeria*. Macrophages and giant cells immunolabeled for this antigen were distributed underneath ulcers, along fissures, around abscesses, within the lamina propria, in granulomas, and in the germinal centers of mesenteric lymph nodes. In addition, 57% (12 of 21) of the cases contained the *E. coli* antigen, and 44% (7 of 16) contained the streptococcal antigen. The immunolabeling for the latter two agents also occurred within macrophages and giant cells, distributed in a pattern similar to that of *Listeria* antigen. Conclusions: The results suggest that *Listeria* spp., *E. coli*, and streptococci, but not measles virus, play a role in the pathogenesis of Crohn’s disease.

Infectious agents have been suspected in the initiation of Crohn’s disease (CD) since this disorder was first recognized. A variety of microbial agents including *Yersinia*, *Brucella*, Campylobacter, mycobacteria, *chlamydia*, *cytomegalovirus*, and herpesvirus have been considered putative agents; however, there is no convincing evidence for the etiologic involvement of any. Several approaches have been used in the attempt to show that microbial agents play a role in the etiology of CD. They include (1) demonstration of the presence of organisms or specific antigens in affected tissues; (2) culture of the microbes from the affected tissues; (3) demonstration of serological responses to the agent; and (4) localization and detection of individual pathogen-specific nucleic acid sequences in affected tissues by in situ hybridization and polymerase chain reaction. The objective of the present study was to use immunocytochemistry to seek selected infectious agents or antigens in diseased tissues. Intestinal tissues and mesenteric lymph nodes from 21 patients were studied. Twelve of the patients were from two French families with an extraordinarily high frequency of CD, and 9 patients were from Connecticut. Tissues of the French patients were previously examined by immunocytochemistry for the antigens of mycobacteria and *Yersinia* and were found to contain none.

Cartun et al. reported that, of 16 cases of CD, *Escherichia coli* and streptococcal antigens were identified in 11 (69%) and 10 (63%), respectively. They suggested that *E. coli* and streptococcal antigens may contribute in a secondary way to the pathogenesis. In the current study, antibodies to these two agents were applied to tissues of a new series of patients. Gump found that the levels of circulating antibodies against *Bacteroides vulgatus* were elevated in patients with CD. This is the same species of *Bacteroides* shown by Breeling et al. to provide immune enhancement of experimentally induced disease in a guinea pig model of inflammatory bowel disease. Therefore, evidence of in situ participation by *B. vulgatus* was examined in the present study. *Listeria monocytogenes* has been occasionally found in the gastrointestinal tract of healthy persons and has been implicated in causing granulomatous hepatitis in animals and humans. Because granulomas are an important feature of CD, *L.
monocyogenes was also sought. Antibody against *Borrelia burgdorferi* was used because of an anecdotal report of the coexistence of CD and Lyme disease in 1 patient.

Multifocal inflammation of the intestinal microvasculature has recently been shown in CD. Wakefield et al. suggested that vasculitis is an early event and hypothesized that the disease is caused by a persistent viral infection of the mesenteric microvascular endothelium. An electron microscopic study by the same group showed paramyxovirus-like particles in tissues of CD, and further studies using in situ hybridization and immunocytochemistry suggested that the organism may be measles virus. In the present immunocytochemical study, antibodies against three paramyxoviruses, measles, parainfluenza, and respiratory syncytial virus (RSV), were used.

The aphthous ulcer, a necrosis of M cells in the epithelium overlying intestinal lymphoid follicles, is regarded as the earliest lesion of CD and the first lesion to recur after surgery. Bovine viral diarrhea virus (BVDV), an RNA virus belonging to the Togaviridae, genus pestivirus, has a high affinity for lymphoid tissues, especially those of the tonsils and Peyer’s patches. Moreover, pestivirus antigen has been found in the feces of children. Therefore, antibody against BVDV was included in the present study. Because influenza virus infection represents a risk factor for CD and its antibody titer is our understanding that all the tissues studied in this study, from patients and from controls, were promptly processed into paraffin blocks at the time of initial diagnostic pathological studies.

The background of two French families with an extraordinarily high frequency of CD was previously reported by Van Kruiningen et al. Briefly, in the first family, both husband and wife and their 4 children were affected. In addition, a spouse of one of the children has recently contracted CD after an 7-year relationship. In the second family, 7 of 11 children have CD. There was neither history of CD in antecedent generations nor consanguinity or linkage to HLA haplotypes in either family.

Formalin-fixed control tissues were either (1) intestinal tissues from patients undergoing colectomy for ulcerative colitis (UC) with either active disease or complications requiring surgery (n = 8); (2) intestinal tissues from patients undergoing surgery for active indeterminate colitis (n = 2); (3) normal intestinal tissues from patients undergoing surgery for intestinal adenocarcinoma (n = 6); (4) tissues from patients with active inflammatory or ischemic intestinal conditions other than CD and UC (n = 8). All control tissues were from the files of the Department of Pathology, Yale University.

### Primary Antibodies

| Organism       | Type     | Code no. | Source           | Dilution |
|----------------|----------|----------|------------------|----------|
| *B. vulgatus*  | PAb      | —        | Onderdonk*       | 1:30,000 |
| *B. burgdorferi* | PAb      | 0301     | ViroStat*        | 1:20,000 |
| *Enterococcus* | PAb      | 2743-90  | DIFCO*           | 1:10,000 |
| *E. coli*      | PAb      | B357     | DAKO*            | 1:5000   |
| *E. coli* (H7) | PAb      | 2159-47  | DIFCO*           | 1:10,000 |
| *L. monocyogenes* | PAb     | 2302-50  | DIFCO*           | 1:50,000 |
| Streptococcus  |          |          |                  |          |
| (group F)      | PAb      | 2697-50  | DIFCO*           | 1:7000   |
| BVDV           | MAb      | —        | Dubovi*          | 1:30,000 |
| Influenza A    | PAb      | —        | NVSL*            | 1:20,000 |
| Measles (NP)   | PAb      | —        | Wakefield*       | 1:2000   |
| Messes (NP)    | MAb      | 16BB8    | Norby*           | 1:10,000 |
| Parainfluenza  | (types 2 and 3) | PAb   | AB1070 | Chemicon* | 1:5000 |
| RSV            |          |          | AB1128 | Chemicon* | 1:30,000 |

NP, nucleocapsid protein; PAb, polyclonal antibody; MAb, monoclonal antibody.

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**Materials and Methods**

**Tissue Sources**

Thirty-eight fixed tissue blocks from 21 patients with CD were studied. Of 29 formalin-fixed tissue blocks from 16 patients, 20 were from 7 patients in two French families and 9 blocks were from 9 patients from the files of the Department of Pathology, Yale University. In each of the 7 French family patients, 3 tissue blocks were examined as follows: (1) the junction between normal and diseased regions was chosen as representative of an early lesion; (2) a region containing important diagnostic elements such as mucosal ulceration, transmural lymphocytic inflammation, fissures, abscesses, and granulomas was chosen as an advanced lesion; and (3) mesenteric lymph nodes were also included, except for 1 case in which they were not available. Nine blocks of intestine and mesenteric lymph nodes fixed in Bouin’s solution were from 5 additional affected members of the two French families. Because positive control tissues of *E. coli*, parainfluenza virus, and RSV with the same fixation were available, studies were also performed using tissues fixed in Bouin’s solution to search for these antigens. Some antigens are rendered undetectable by immunocytochemistry when stored for a long time in formalin, a change that could produce false negative results. It is our understanding that all the tissues studied in this study, from patients and from controls, were promptly processed into paraffin blocks at the time of initial diagnostic pathological studies.

The background of two French families with an extraordinarily high frequency of CD was previously reported by Van Kruiningen et al. Briefly, in the first family, both husband and wife and their 4 children were affected. In addition, a spouse of one of the children has recently contracted CD after a 7-year relationship. In the second family, 7 of 11 children have CD. There was neither history of CD in antecedent generations nor consanguinity or linkage to HLA haplotypes in either family.
Enterococcus, L. monocytogenes types 1 and 4, Streptococcus group F, BVDV, influenza A virus, measles virus, parainfluenza virus, and RSV, were used. The optimal dilution and source of each antibody are summarized in Table 1. The antibodies against E. coli, Enterococcus, and Streptococcus group F were the same as those previously applied by Cartun et al. in a different series of patients. The antibody to Listeria was raised against the O antigen, and the antibodies to E. coli and Streptococcus group F were raised against polysaccharides.

**Positive Control Tissues**

Control tissues containing specific pathogens were chosen from naturally infected animals or humans. The presence of bacteria in the control tissues was confirmed by culture and presence of virus by clinical and pathological features. For bacterial pathogens for which naturally infected tissues were not available, suspensions of cultured bacteria were injected into pieces of normal tissues (intestine or lung) or mixed with minced pieces of normal kidney or lung before routine fixation, paraffin embedding, and sectioning. Brown–Hopps modified Gram stain or modified Steiner's silver stains were used to confirm the presence of bacteria in these tissues. For the viruses, commercially available virus-infected cultured cells were fixed in formalin or Bouin's solution, embedded in paraffin, and sectioned as positive controls. Source details for each of these positive controls are shown in Table 2.

**Confirmation of Antibody Specificity**

All antibodies used in this study were tested by immunocytochemistry for cross-reactivity. Each antiviral or antibacterial antibody was applied to cells or tissues that included all the positive controls for each group (viruses or bacteria). In addition, polyclonal L. monocytogenes antibody was also evaluated for cross-reactivity against Brucella, Mycobacterium paratuberculosis, Salmonella, Streptococcus group B, and Yersinia pseudotuberculosis. All antibodies against viruses used in this study were also tested for possible cross-reaction to coronavirus and canine distemper virus. There were no immunocytochemical cross-reactions among bacterial antibodies examined. Among antibodies against viruses, cross-reaction occurred only between antibodies against measles virus and canine distemper virus.

**Detection System**

Indirect and the labeled streptavidin-biotin-peroxidase immunocytochemical techniques were similar to those used by Cartun et al. Paraffin sections were cut by microtome at 5 μm and placed on slides coated with poly-L-lysine or aminosilane to promote section adherence. Tissue sections were deparaffinized in xylene, rehydrated through graded alcohols, and rinsed in tap water. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 5 minutes. The tissue was then rinsed with tap water and incubated in phosphate-buffered saline (pH 7.4) for 5 minutes, and the primary antibody at predetermined optimal dilution was applied. Slides were placed in humidity chambers and incubated for 16 hours at 4°C. For the labeled streptavidin-biotin-peroxidase method, biotinylated secondary antibody appropriate to the primary antibody was added and incubated at room temperature for 30 minutes. The sections were further covered with streptavidin horseradish peroxidase conjugate after the excess secondary antibody was rinsed off. After additional washings, the sections were covered with amino-ethylcarbazole chromogen and incubated at room temperature for 20 minutes. The chromogen was rinsed from the slides in running tap water for 1 minute, and the tissues were counterstained with Lerner-2 hematoxylin for 45 seconds, with amino-ethylcarbazole chromogen and incubated at room temperature for 20 minutes. The chromogen was rinsed from the slides in running tap water for 1 minute, and the tissues were counterstained with Lerner-2 hematoxylin for 45 seconds, with amino-ethylcarbazole chromogen and incubated at room temperature for 20 minutes. The chromogen was rinsed from the slides in running tap water for 1 minute, and the tissues were counterstained with Lerner-2 hematoxylin for 45 seconds, with amino-ethylcarbazole chromogen and incubated at room temperature for 20 minutes. The chromogen was rinsed from the slides in running tap water for 1 minute, and the tissues were counterstained with Lerner-2 hematoxylin for 45 seconds, with amino-ethylcarbazole chromogen and incubated at room temperature for 20 minutes.

**Table 2. Positive Control Tissues**

| Organism | Source of organisms and tissues |
|----------|---------------------------------|
| B. vulgatus | Minced human kidney mixed with organisms (BV 4062-33) provided by Dr. A. Onderdonk at Harvard Medical School |
| B. burgdorferi | Minced human kidney mixed with organisms from Dr. S. Buchmich at University of Connecticut |
| E. coli | Normal colon from a surgical resection of adenocarcinoma, injected with organisms (ATCC 25922) |
| E. coli (H7) | Normal colon from a surgical resection of adenocarcinoma, injected with organisms (ATCC 43868) |
| L. monocytogenes | Infected ovine brain confirmed by pathological features and microbiological culture |
| Mycobacteria | Intestine from a cow with Johne's disease (M. paratuberculosis) confirmed by clinical signs, pathological features, and acid-fast staining |
| Salmonella | Normal colon from a surgical resection of adenocarcinoma, injected with Salmonella enteritidis (ATCC 29628) |
| Streptococcus spp. | Normal colon from a surgical resection of adenocarcinoma, injected with organisms (ATCC 19433, ATCC 9809, and ATCC 12393) |
| Yersinia | Lung injected with Y. pseudotuberculosis (ATCC 907) |
| BVDV | Experimentally infected bovine brain |
| CDV | Naturally infected raccoon brain and intestine confirmed by pathological features |
| Coronavirus | Naturally infected bovine intestine confirmed by clinical signs, pathological features, and electron microscopy |
| Influenza A | Naturally infected avian lung, kidney, and trachea confirmed by clinical signs and immunocytochemistry by Dr. E. Swayne at Ohio State University |
| Measles | a. Virus-infected HEP-2 cell from Bion Inc., Park Ridge, IL |
| | b. Subacute sclerosing panencephalitis: paraffin sections of human brain from autopsy of clinically and serologically proven case |
| Parainfluenza (types 2 and 3) | Virus-infected Vero cells from Bion Inc. |
| RSV | Virus-infected Vero cells from Bion Inc. |

CDV, canine distemper virus.
jugated secondary antibody was used. After a 30-minute incubation at room temperature, sections were treated with aminobenzylcarbazole chromogen as in the former method.

Normal serum (5%, vol/vol) from the species of animal in which the secondary antibody was produced was applied to block nonspecific reactivity. Appropriate positive controls and negative controls, with no primary antibody applied, were run in parallel. The immunostained slides were initially examined by one investigator with knowledge of the case histories, and then all positive slides were reexamined with a second and sometimes third investigator using a multihheaded microscope.

Results

Bacterial Antigens

No immunolabeling for B. vulgatus, B. burgdorferi, or E. coli H7 was present in any of the tissues studied. In contrast, 12 of the 16 CD cases (75%) were immunolabeled by Listeria antiserum (Figure 1). The positive labeling for this antigen occurred in the cytoplasm of macrophages, most in granulomas, which were distributed underneath ulcers (8 of 12), along fissures (5 of 12), in the lamina propria (1 of 12), around abscesses (1 of 12), and in the germinal centers of mesenteric lymph nodes (1 of 12). In addition, giant cells were labeled in 3 of these 12 cases; they were distributed along fissures, beside an abscess, or in granulomas. None of the 10 normal control cases, 2 indeterminate colitis cases, and 8 cases with other forms of enterocolitis showed positive immunolabeling with L. monocytogenes antibody. One of 8 UC cases had positively labeled macrophages in the lamina propria. Despite intensive searching, Gram-positive rods could not be shown in the areas where positive L. monocytogenes immunolabeling occurred. When Listeria antiserum was first neutralized by incubation with cultured Listeria organisms and then applied to serial sections of all antigen-positive CD tissues, no positive staining was found in any of these tissues.

In 12 of 21 cases of CD (57%), tissues were positively labeled by polyclonal E. coli antibody. All 12 had positively labeled macrophages underneath ulcers, along fissures, and in the mucosal lamina propria, and 4 cases had positively labeled giant cells along fissures, underneath ulcers, and in granulomas in the submucosa and subserosa (Figure 2). Moreover, some immunolabeling was also distributed on the surface of ulcers and fissures. None of the tissues from the four groups of controls had positive labeling with polyclonal E. coli antibody.

Group F Streptococcus immunoreactivity occurred in 7 of 16 (44%) CD cases. Of these, 1 had positively labeled macrophages underneath ulcers, 2 showed positive macrophages and giant cells along fissures (Figure 3A), 1 had positive macrophages in an abscess, 2 had positive macrophages in the lamina propria, and 1 had positive macrophages and giant cells in the germinal centers of a mesenteric lymph node (Figure 3B). Group F Streptococcus immunoreactivity was present in the lamina propria in 1 of 10 normal (10%), and 2 of 8 UC controls (25%). One of 2 (50%) indeterminate colitis cases had labeled macrophages along fissures. None of the 8 cases with other forms of enterocolitis had immunoreactivity with group F Streptococcus antibody. Positive staining for Enterococcus was found in 2 of 16 (13%) CD cases, with the positive macrophages occurring along fissures. No immunoreactivity to Enterococcus was found in any of the control cases.

In general, the distribution of Listeria antigen was more aggregated than that of E. coli and streptococci. In the 16 CD cases from which formalin-fixed tissues were available, 2 had positive labeling for only one bacterial antigen, 11 had multiple antigens labeled but not in the same cells, and 3 were negative for all bacterial antigens. Results of immunocytochemistry from each case are shown in Table 3. Results are compared with control groups in Table 4.

Viral Antigens

No immunolabeling of BVDV, influenza A, parainfluenza, or RSV was present in any case examined. In all the cases studied, including 16 patients with CD, 10 normal controls, and 8 patients with UC, tissues were nonspecifically labeled by polyclonal antibody. The immunolabeling occurred in the nuclei of macrophages, fibroblasts, giant cells, smooth muscle cells, and nerves. The labeled cells were distributed in the lamina propria, submucosa, muscle layers, subserosa, and granulomas (except that neither granulomas nor giant cells occurred in the normal or UC tissues). Of 16 patients with CD, 3 showed similar labeling of nuclei in endothelial cells of small vessels. In contrast to these results with polyclonal antibody, none of the CD, UC, and normal control tissues were labeled by monoclonal measles nucleocapsid protein antibody, although highly specific staining with this antibody was observed in control tissue of subacute sclerosing panencephalitis.

Discussion

In the present study, antibody to L. monocytogenes seemed to identify antigen in 75% of CD cases studied. To our knowledge, this is the first suggestion that Listeria may occur in intestinal tissues of patients with CD. The demonstration of positively labeled macrophages and giant cells underneath ulcers, along fissures, in the lamina propria, in abscesses, and in the mesenteric lymph nodes suggests that the antigen labeled is important in CD.
Figure 1. Immunocytochemical labeling with *L. monocytogenes* antibody using streptavidin-biotin-peroxidase technique. (A) Immunolabeled *Listeria* organisms (rust red) in a microabscess of positive control tissue from an ovine brain. (B) Immunoreactivity in macrophages (rust red) along a fissure in the colon from a patient with CD (case 9). (C) Positively labeled macrophages surrounding a microabscess in the colon from a patient with CD (case 17). (D) Immunoreactivity in a multinucleate giant cell and macrophages at the base of a transmural fissure in the terminal ileum from a patient with CD (case 9) (Lerner-2 hematoxylin counterstain; original magnification 640x).
Figure 2. *E. coli* immunoreactivity in tissues from patients with CD, labeled by streptavidin-biotin-peroxidase technique. (A) Two multinucleated giant cells along a fissure in the terminal ileum (case 4). (B) Immunolabeled multinucleated giant cells and macrophages (arrows) in a subserosal granuloma in the terminal ileum (case 4) (Lerner-2 hematoxylin counterstain; original magnification 640×).

Figure 3. Group F streptococcal immunoreactivity in tissues from patients with CD, labeled by streptavidin-biotin-peroxidase technique. (A) Immunoreactivity within giant cells and macrophages along a fissure in terminal ileum (case 9). (B) Immunolabeled histiocytes in the germinal center of a mesenteric lymph node (case 6) (Lerner-2 hematoxylin counterstain; original magnification 640×).
Although it is possible that the \textit{L. monocytogenes} antiserum used may cross-react with some intestinal microflora, other pathogenic bacteria, or other species of \textit{Listeria}, our results clearly exclude any cross-reaction with \textit{E. coli}, \textit{B. vulgatus}, \textit{B. burgdorferi}, \textit{Brucella}, \textit{Enterococcus}, \textit{M. paratuberculosis}, \textit{Salmonella}, group B and F \textit{Streptococcus}, and \textit{Y. pseudotuberculosis}.

\textit{Listeria} are widely distributed in nature and have been isolated from fecal specimens of healthy ferrets, chinchillas, ruminants, and humans. Human and animal populations can be healthy gastrointestinal carriers of this organism.\cite{16} In an animal model, \textit{L. monocytogenes} was able to colonize the gastrointestinal tract of germ-free mice, cross the gut mucosa, and cause systemic infection.\cite{19} \textit{L. monocytogenes} are facultative intracellular pathogens, capable of producing granulomatous reactions. Granulomas and microabscesses have been shown in a liver biopsy specimen from an adult patient with listeriosis.\cite{18}

MacDonald and Carter\cite{31} reported that the oral administration of \textit{L. monocytogenes} consistently initiated infection in the Peyer’s patches of the small intestine of SPF B6D2F1 mice. The Peyer’s patches appeared to be the only site where \textit{L. monocytogenes} made an initial invasion and survived in the intestinal tissues. It is tempting to suggest that on interacting with the host mucosa, \textit{Listeria} may induce the aphthous ulcer, regarded as the earliest lesion of \textit{CD}\cite{27} and the first to recur after surgery.\cite{25} On the other hand, \textit{L. monocytogenes} antigen was coexistent with \textit{E. coli} or streptococcal antigen in 83\% of the patients with \textit{CD} containing labeled antigen (10 of 12), suggesting that \textit{L. monocytogenes} may act as a secondary invader. \textit{Listeria} antigen was not colocalized in the same cells or aggregates of cells. Gram staining of foci of immunoreactivity did not show intact bacterial forms, raising the possibility that organisms responsible for the immunolabeling have been degraded within phagocytic cells, or perhaps occur in L-form. Blaser et al. found no significant increase in the mean of anti-\textit{Listeria} antibody titers in patients with \textit{CD} (\textit{n} = 40) compared with age- and sex-matched healthy controls (\textit{n} = 40).\cite{2} Further serological studies as well as assays of cellular immunity, polymerase chain reaction, and microbiologic culture are needed to elucidate the role of \textit{L. monocytogenes} in this disease.

In the present study, \textit{E. coli} antigen was shown in 57\% (12 of 21) of patients with \textit{CD}. The immunoreactivity was observed in both macrophages and giant cells, distributed underneath ulcers, along fissures, in granulomas, and in the lamina propria. This is consistent with the work of Cartun et al.\cite{11} who found \textit{E. coli} antigen in 69\% of 16 \textit{CD} cases. Our results are also in agreement with the serological findings of Brown and Lee\cite{32} and Tabaqchali et al.\cite{33} who showed that \textit{E. coli} antibody titers were higher in patients with \textit{CD} than in controls. In addition, \textit{E. coli} and other potentially pathogenic bacteria were isolated more frequently from the intestinal serosa and mesenteric nodes of patients with \textit{CD} (27\% and 33\%) than in controls.\cite{34} In the study of Tabaqchali et al.,\cite{33} sera from 30 patients with inflammatory bowel disease including 16 with \textit{CD} and 14 with UC were assayed for the presence of antibodies against 159 \textit{E. coli} O antigens and compared with sera from 16 matched control subjects. The patients with inflammatory bowel disease had higher titers of antibody than the control group, but there was no single serotype that was associated with the disease, suggesting that the \textit{E. coli} participation was secondary.

Group F \textit{Streptococcus} immunolabeling was found in 44\% of patients with \textit{CD}, another finding consistent with the results of Cartun et al.\cite{11} Injection of streptococcal cell wall fragments into the small intestine and cecum
has been shown to induce granulomatous enterocolitis in rats. Streptococcal lipoteichoic acid can be carried by the blood to other body sites. Streptococci may have an important role in inducing extraintestinal features of CD. Streptococcal immunoreactivity was also found in 25% of UC cases, 10% of normal intestine, and 1 of 2 indeterminate colitis cases. Streptococci more than likely act as secondary rather than primary agents.

On the basis of immunolabeling of formalin-fixed tissues, 11 of 16 patients with CD had antigen of more than one kind of bacteria. The involvement of multiple bacteria can be explained in the following ways. First, the disease may be caused by several microbial agents acting in sequence. Second, a predisposing genetic susceptibility, an environmental agent, or a specific virus infection may cause increased intestinal permeability that results in invasion by more than one bacterium. Third, this may represent colonization and invasion of ulcerated bowel by several members of the resident flora.

In this study, nonspecific immunoreactivity to polyclonal measles antibody occurred in all CD, UC, and normal control tissues, with almost all of the immunolabeling occurring in the nucleus. In contrast, none of the tissues were immunolabeled by monoclonal measles nucleocapsid protein antibody, even though the positive controls (cell cultures containing measles virus and tissues from a patient with subacute sclerosing panencephalitis) were clearly and specifically labeled by the latter, both in the nucleus and in the cytoplasm. Because the polyclonal measles antiserum was not affinity purified, it appears that it may contain some antibody that is reactive with normal cellular components. Measles virus phosphoprotein is known to have immunologic cross-reactivity for other paramyxoviruses and found no evidence of parainfluenza or RSV in CD tissues, particles that have the size and morphology of paramyxoviruses. Because we could not show evidence of measles virus, we used immunocytochemistry to search for other paramyxoviruses and found no evidence of parainfluenza or RSV. The identity of the viralike particles remains to be determined. Additional immunocytochemistry and immunoelectron microscopy are needed to further explore the role of viruses in the initiation of CD.

In summary, our results do not support a role for measles virus, parainfluenza virus, RSV, BVDV, B. vulgatus, or B. burgdorferi in the etiology or pathogenesis of CD. They confirm the presence of antigens of one or more bacteria in most cases of CD, typically that E. coli and Streptococcus spp. are consistently present. In addition, we have shown for the first time that the lesions of a majority of patients (75%) with CD, from two separate populations in Europe and North America, contain important antigen-bearing macrophages and giant cells that have immunoreactivity with antibody to L. monocytogenes. The specificity and significance of this latter finding remain to be determined.

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Address requests for reprints to: Herbert J. Van Kuileningen, M.D., Department of Pathobiology, University of Connecticut, Box U-89, 61 North Eagleville Road, Storrs, Connecticut 06269. Fax: (203) 486-2794.

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