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Experimental *Campylobacter jejuni* Infection in Humans

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Two strains of *Campylobacter jejuni* ingested by 111 adult volunteers, in doses ranging from 8×10^2 to 2×10^9 organisms, caused diarrheal illnesses. Rates of infection increased with dose, but development of illness did not show a clear dose relation. Resulting illnesses with strain A3249 ranged from a few loose stools to dysentery, with an average of five diarrheal stools and a volume of 509 mL. Infection with strain 81-176 was more likely to cause illness, and these illnesses were more severe, with an average of 15 stools and 1484 mL of total stool volume. All patients had fecal leukocytes. The dysenteric nature of the illnesses indicates that the pathogenesis of *C. jejuni* infection includes tissue inflammation. Ill volunteers developed a serum antibody response to the *C. jejuni* group antigen and were protected from subsequent illness but not infection with the same strain.

Campylobacter jejuni is now recognized as an important cause of acute diarrheal disease throughout the world. Although a few infections with this organism were identified as long as 25 y ago, it was not until the development of selective stool-culture techniques in 1972 that its importance was appreciated [1]. Since that time, studies in developed countries (such as Belgium, the United Kingdom, Australia, Canada, the Netherlands, Sweden, and the United States) have demonstrated *C. jejuni* in the stools of 4%–14% of patients with diarrhea and of <1% of asymptomatic persons [2]. Studies in developing countries (such as Bangladesh, Peru, Rwanda, and The Gambia) suggest that *C. jejuni* may be even more commonly isolated during diarrheal illness than in developed countries but that the rate of asymptomatic infection is often also very high [2–4].

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Informed consent was obtained for all studies, which were approved by the ethical review committees of the University of Maryland School of Medicine and the National Institute of Allergy and Infectious Diseases.

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This high rate of infection without illness has led some investigators to question whether all strains of *C. jejuni* are equally pathogenic or to speculate that acquired immunity protects from illness but not stool colonization [5, 6]. Diarrhea has been induced in rhesus monkeys by oral inoculation of *C. jejuni* [7], and two human volunteers became ill after swallowing *C. jejuni* isolated from a patient with disease [8, 9]. The broad clinical spectrum, ranging from a few loose stools to watery diarrhea to dysentery, reported with *C. jejuni* infection and the identification of a variety of possible virulence properties (such as cytotoxins, enterotoxins, or "invasiveness"), have led to questions regarding the pathogenesis of the illness and the relation of clinical manifestations to these properties [10]. The lack of a universally accepted small animal model that is representative of natural infection has impeded better understanding of these relations.

Thus, volunteer studies in American adults were initiated to establish certain basic features of the infection and illness, including the quantity of organisms needed to cause illness, the pathogenesis of the illness, the development of an immune response to infection, the development of illness, and the extent of homologous immunity after illness.

Subjects and Methods

Volunteers. Participants were healthy, young adults from the Baltimore community. The methods of medical screening, informed consent, and medi-

cal care have previously been published [11–13]. Persons with HLA allotype B27 were specifically excluded from the studies because of reports of occasional arthritis occurring after *C. jejuni* infection in individuals with this allotype.

Challenge studies. Volunteers were admitted to the isolation ward and challenged with *C. jejuni* suspended in 150 mL of milk (reconstituted from dry skim-milk powder). In one study the organisms were given in a suspension containing 2 g of sodium bicarbonate instead of in milk. Volunteers fasted for 90 min before and after ingesting the organisms. After challenge they were interviewed and examined daily for 12 d by a physician. All stools were collected in a sterilizable plastic pan that fit on the commode, examined by a staff member, graded for consistency [13], weighed, and tested for blood by Hemoccult® (Smith Kline Beckman, Sunnyvale, Calif). Diarrhea was defined as the passage of two or more liquid stools, weighing at least 200 g, within 48 h or of a single liquid stool weighing at least 300 g or containing blood. Temperatures were taken every 6 h; any temperature ≥37.8 C was repeated again in 5 min with a different thermometer. Illness was considered the presence of diarrhea or fever (temperature >38 C). Volunteers with diarrhea received oral glucose-electrolyte solutions to prevent acidosis and volume depletion. All volunteers were observed for seven days before beginning treatment with erythromycin stearate (250 mg every 6 h for five days).

All stools passed after challenge until discharge from the study were cultured. If no stool was passed in a 24-h period, a rectal swab was obtained for culture. After discharge, stool samples for culture were obtained on days 14, 21, and 28. A wet mount of each stool sample was made on a glass slide, stained with methylene blue, and the number of white blood cells per high-power field was counted by an experienced technician who did not know the illness status of the volunteers. Cultures of blood were done for all volunteers at 30–60 min and at 12, 24, 48, 72, 96, 120, and 144 h after challenge. Jejunal fluid for culture was collected by having volunteers ingest a gelatin capsule string device (Entero-Test®; Health Development, Palo Alto, Calif.) at intervals of 20 and 44 h after challenge and by withdrawing the string for culture after 4 h. For three ill patients, sigmoidoscopy and rectal biopsy were performed.

Bacterial strains. Two *C. jejuni* strains were used in these challenge studies. Strain A3249 (Penner serotype 27) was isolated from a 16-y-old boy with a sporadic infection [14] after an outbreak at a camp in Connecticut [15]. The patient had had a two-day illness with several loose stools, headache, nausea, and a temperature of 38 C. Strain 81–176 (Penner serotype 23/36) was isolated from an ill nine-year-old girl in an outbreak in Minnesota in which 52% of those consuming the implicated raw milk developed symptoms [16]. In this outbreak, all isolates were Penner serotype 23/36, and of the 25 ill persons, 100% had diarrhea, 84% had abdominal cramps, and 72% were febrile; none had grossly bloody stools. Strain A3249 manifested two colony types, spreading and nonspreading, which represent flagellated and aflagellated variants (Dr. H. Lior, personal communication). Both strains were evaluated in the removable intestinal tie adult rabbit diarrhea (RITARD) model, in which 10^9 organisms were injected into the mid-jejunum [17]. Strain A3249, but not strain 81-176, was considered invasive; however, neither strain was found to be enterotoxigenic by using the methods of Klipstein et al. [10; tests done by Dr. F. A. Klipstein]. Strain 81-176 has been found to produce Shiga-like toxin, although at titers less than one-thousandth of those found for *Shigella dysenteriae* type 1 [18, 18a], and is susceptible to the bactericidal activity present in normal human serum [19]. In total, each strain had been passaged five to 10 times on artificial media before being given to volunteers.

Challenge inocula. Stock cultures of *C. jejuni* that had been maintained in glycerol stored at −70 C were subcultured onto blood agar or Mueller-Hinton agar plates and incubated at 42 C in an atmosphere of 6% oxygen and 10% carbon dioxide by using an anaerobic jar and Campy Pak II® (BBL Microbiology Systems, Cockeysville, Md). From the plate, 40–50 colonies were suspended in thioglycolate soy broth, and this suspension was plated onto Mueller-Hinton agar. After incubation overnight at 42 C, the bacterial culture was harvested with 5 mL of PBS, diluted with PBS to approximate the number of organisms required for challenge, and standardized spectrophotometrically. Replicate pour-plate quantitative cultures of the inocula were made before and after challenge to confirm inoculum size. The final inoculum was examined by gram strain and was agglutinated with specific antiserum before ingestion by volunteers. Because strain A3249 manifested two colony types, spreading and nonspreading, separate challenge inocula were prepared to equally represent these two types in the final inoculum. For example,

with strain A3249, an inoculum of 8×10^3 was comprised of 4×10^3 of each colony type.

Bacteriology. Stool specimens, rectal swabs, and the fluid from Entero-Test strings were plated onto Campy-BAP® (BBL Microbiology Systems) containing brucella agar, 10% sheep erythrocytes, and the following antimicrobial agents per liter: vancomycin, 10 mg; trimethoprim, 5 mg; polymyxin B, 2500 IU; amphotericin, 2 mg; and cephalothin, 15 mg. Plates were incubated at 42 C for 18–24 h. Colonies with the appearance of *C. jejuni* were smeared and stained with 0.2% carbol fuschin, and the organisms were confirmed by motility and by oxidase and catalase positivity. Isolates from the stools of 19 infected volunteers were serotyped by Dr. J. Penner (University of Toronto, Toronto).

Blood was cultured by two methods. Two milliliters of blood was mixed in a tube with 18 mL of thioglycolate broth, then incubated at 42 C in an anaerobic jar with Campy Pak II (BBL Microbiology Systems) for 48 h. After 48 h the broth was streaked onto brucella agar and all organisms identified. Four milliliters of blood was put into each of the two Bactec (aerobic and anaerobic) bottles (Bactec Systems, Johnston Laboratories, Cockeysville, Md) and processed by the University of Maryland Hospital laboratory by using standard methods [20, 21].

Serology. Serum samples from 44 volunteers challenged with 8×10^2–8×10^5 cfu of *C. jejuni* strain A3249 were examined for IgA, IgG, and IgM antibodies specific for *C. jejuni*. Sera were collected before challenge and at 11, 21, and 28 d later. The ELISA, using *C. jejuni* group-specific surface proteins as the antigen and immunoglobulin class-specific peroxidase conjugates, has been described in detail [22, 23]. Because analysis of serially diluted test sera has shown a linear relation between the optical density value determined and reciprocal titer [22], all sera were assayed at single screening dilutions: 1:50 for IgA and IgM and 1:100 for IgG determinations. These dilutions were selected for the ability of the assays to distinguish between persons with known natural, acute *C. jejuni* infections and uninfected persons [22].

Statistical analysis. Clinical comparisons were done by χ^2, Fisher's exact test, and group *t* test (two tailed). For analysis of differences in antibody response over time within a single group (defined by clinical response to infection), paired *t* tests were used. Because postchallenge antibody levels were expected to be higher than initial levels, these tests were interpreted in a one-tailed fashion. For analysis of differences between groups, independent *t* tests were used. Because there was no a priori expectation of the direction of differences, these tests were interpreted in a two-tailed fashion.

Results

RITARD results. Of seven rabbits given strain A3249 in the RITARD model, all became infected and ill; three died. Of eight rabbits given strain 81-176, all became infected and ill; three died.

Challenges with strain A3249. Six studies were done to establish the relation between the ingested dose of *C. jejuni* strain A3249 and the rates of infection and illness (table 1). These studies demonstrated that the rate of infection increased from 50% to 100% as the inoculum was raised from 800 to 10^8 cfu. Although illness resulted from the lowest dose ingested (800 total or 400 flagellated *C. jejuni*), the attack rate did not increase consistently with higher inocula, nor did the incubation period or severity of illness appear to differ by the size of the inoculum. To determine if the relatively low attack rates with strain A3249 were due to inadequate or variable neutralization of gastric acid by the milk ingested with the challenge inoculum, we compared giving the inoculum in milk to giving it with 2 g of sodium bicarbonate. Nine volunteers given 10^8 organisms by either method became infected. None of the five given the organisms with milk became ill, but two of the four given organisms with bicarbonate developed diarrhea.

Stool cultures usually become positive by the second to third day after challenge and stayed positive until 24–48 h after erythromycin treatment was begun. Despite inoculation with equal numbers of organisms of the spreading and nonspreading colony types of strain A3249, stool cultures of infected volunteers had only the spreading colony type. Isolates from the stools of 19 volunteers were type 27, the serotype of the challenge strain. No string cultures were positive with ingested doses of $<10^6$ organisms. With a dose of 10^6 cfu, only 16% were positive at 24 h after challenge (none were positive at 48 h). With 10^8 cfu, 60% were positive at 24 h and 20% at 48 h. Cultures of blood after challenge (532 sets) were negative by both Bactec and thioglycolate techniques.

Overall, 13 (18%) of 72 individuals given strain A3249 became ill; 12 had diarrhea (4 also had fe-

Table 1. Clinical and bacteriologic results of challenging healthy adults with *C. jejuni* strains A3249 and 81-176.

Strain, dose	No. of volunteers			Percentage of volunteers		Mean	
	Total	With fever	With diarrhea	With diarrhea or fever	With positive stool cultures	No. of liquid stools	Diarrheal volume (mL)
A3249							
8×10^2	10	1	1	10	50	2.0	106
8×10^3	10	0	1	10	60	4.0	158
9×10^4	13	2	6	46	85	5.3	533
8×10^5	11	0	1	9	73	4.0	302
1×10^6	19	2	1	11	79	16.0	1574
1×10^8	5	0	0	0	100	-	-
1×10^8*	4	0	2	50	100	2.5	388
Total	72	5	12	18	75	5.3†	509†
81-176							
1×10^6	7	2	3	43	100	29.7	2896
2×10^8	10	2	6	60	100	11.0	1092
2×10^9	22	2	9	41	100	12.0	1275
Total	39	6	18	46	100	14.6†	1484†

* All inocula were given with 150 mL of nonfat milk, except this inoculum, which was given with 2 g of sodium bicarbonate.
† $P < .05$, by two-tailed t test, for strain A3249 vs. strain 81-176.

ver), and 1 had only fever. The average time to the onset of fever was 68 h and to the onset of diarrhea, 88.5 h. In general, the diarrhea was mild, with an average of five liquid stools and 0.5 L of stool volume; eight of the 12 volunteers had gross or microscopic blood in the stool, and all had fecal leukocytes. Anorexia, malaise, and abdominal cramps were reported by 50%-60% of ill volunteers. Sigmoidoscopy of three ill volunteers showed normal findings in two, whereas one revealed a diffusely abnormal mucosa, with edema and loss of normal vascular pattern. Microscopic examination of rectal biopsy specimens in the patient with an abnormal mucosa and in one patient with normal findings on sigmoidoscopy showed a mixed population of inflammatory cells, with PMNLs in the crypts and lymphocytes and plasma cells in the muscularis mucosa. There were no relapses after treatment with erythromcyin.

Challenges with strain 81-176. Three studies were done with *C. jejuni* strain 81-176 (table 1). At doses ranging from 10^6 to 10^9 cfu, all volunteers developed a positive stool culture. Stool cultures in ill or well individuals became positive within 72 h and usually remained positive until erythromycin was begun. String cultures of the upper gut were positive in 6 (15%) of 39 persons at 24 h and in only 1 person at 48 h. All cultures of blood (280 sets) were negative.

Overall, 18 (46%) of volunteers challenged with strain 18-176 became ill. No obvious dose-response relation was noted, but the attack rate appeared to be higher than that with strain A3249; 10 (59%) of 17 volunteers receiving 10^6-10^8 cfu of strain 81-176 became ill, versus 4 (14%) of 28 receiving a similar dose of strain A3249 ($P = .0026$, by Fisher's exact test). In the 18 ill individuals, the incubation period was 53 h to the onset of diarrhea and 67 h to the onset of fever. The average illness had 15 stools and nearly 1.5 L of stool loss; three of the cases had >20 liquid stools, and five had a >2-L diarrheal stool loss. These illnesses were significantly more severe than those resulting from strain A3249 (table 1). Fourteen (78%) of the ill persons had blood in their stools, and all had fecal leukocytes. Anorexia, malaise, and abdominal cramps were reported in 67%-78% of ill volunteers. There were no relapses after treatment.

Homologous rechallenge. Homologous protective immunity from prior disease was evaluated in studies with each of the strains. Two volunteers who developed illness with 10^6 strain A3249 *C. jejuni* (veterans) were rechallenged one month after recovery with 10^8 of the same strain, as were five volunteers who had not participated in previous *C. jejuni* volunteer studies (controls). Neither of the two veterans rechallenged with *C. jejuni* became infected after rechallenge, compared with all five controls ($P = .048$); no illnesses occurred. In the second study, seven volunteers who had had illness after receiving 10^8-10^9 cfu of *C. jejuni* strain 81-176 were rechallenged one month later with 10^9 cfu of the same

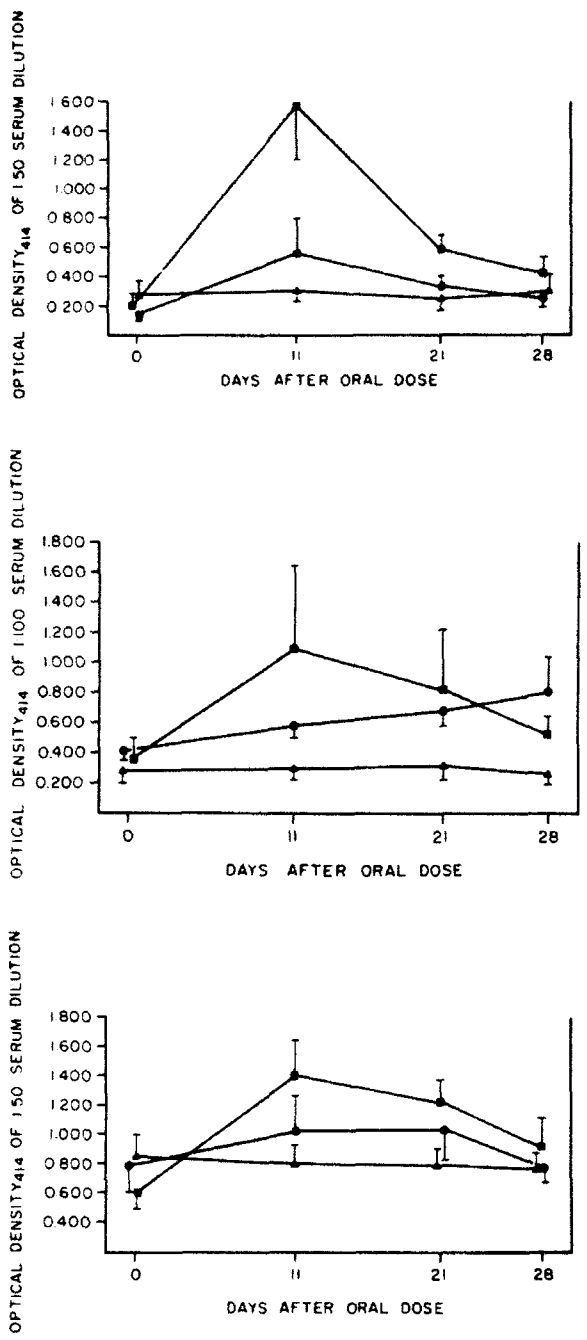

Figure 1. *C. jejuni*-specific antibody response (*top*, IgA; *middle*, IgG; and *bottom*, IgM) in 44 volunteers during four weeks after challenge with *C. jejuni*. Sera were obtained immediately before oral challenge with *C. jejuni* strain A3249 and at days 11, 21, and 28 after challenge. Antibodies to *C. jejuni* surface proteins were detected as described in Subjects and Methods. Each point represents the mean ± SE (*bar*) of the OD values for each group at a given time point. ▲, Not infected ($n=14$); ●, infected – well ($n=21$); and ■, infected – ill ($n=9$).

strain, as were 12 controls. Stool colonization occurred in five of the seven veterans and in all of the controls. Diarrheal illnesses developed in none of the veterans, compared with six of the controls (P = .034). The average illness in controls consisted of 12 liquid stools with 1426 mL of total volume.

Serological response to challenge. There were no significant differences in prechallenge levels of serum antibody in any immunoglobulin class to *C. jejuni* between the groups of volunteers challenged with strain A3249: those who did not show clinical or bacteriologic evidence of infection (not infected), those who were infected but had no symptoms (infected – well), and those who were infected and became ill (infected – ill). Volunteers who were challenged with *C. jejuni* but did not become infected did not show any increase in levels of *C. jejuni*-specific IgA, IgG, or IgM over the 28-d observation period (figure 1). In contrast, for volunteers who were infected and became ill, IgA and IgM levels peaked at 11 d and remained significantly ($P < .05$) elevated for the entire period. IgG response in infected, ill volunteers showed the same trend but did not reach statistical significance ($P < .1$). Rises in levels of serum antibody after challenge in all three immunoglobulin classes were intermediate between those two groups in the infected, well volunteers.

Discussion

These studies indicate that even low doses of *C. jejuni* may produce infection and illness in humans. Lower rates of infection in volunteers receiving low doses and the suggestion in these studies of a higher rate of illness when *C. jejuni* was ingested with sodium bicarbonate rather than milk, may indicate that the organisms may not always survive the barrier of gastric acid [24]. It is also possible that competition by the indigenous flora resulted in sequestration of *C. jejuni*, but the lack of antibody response in these patients suggests lack of infection rather than sequestration. Under natural conditions, low attack rates may be associated with exposure to low doses of organisms and may explain the mostly sporadic nature of these illnesses [25].

The experimental infections produced similar signs and symptoms as those due to naturally acquired infections in the developed countries [26]. Many of the infected volunteers developed fever, blood stools, and fecal leukocytes, although the clinical illnesses in the hosts from whom the strains were

originally isolated were milder. Therefore, the clinical features of a *C. jejuni* infection in an individual case may not provide a full picture of the pathogenic potential of the infecting strain. That both strains were pathogenic indicates that the properties responsible for illness are stable after at least five in vitro passages.

The positive string-tests indicate that *C. jejuni*, which survive and multiply in the presence of bile [24], colonize the upper small intestine early in the course of infection. The positive rectal biopsy demonstrates that the distal colon is a target organ. The presence of small-volume stools and fecal blood and leukocytes suggests that the colon may indeed be the most prominent site of infection. The importance of inflammation in the pathogenesis of the diarrheal illness due to *C. jejuni* is suggested by several observations from this study. First, fever often preceded the onset of diarrhea, as has been found after naturally occurring infections [26]. Second, all ill persons had fecal leukocytes. Third, rectal biopsy specimens from two patients showed inflammatory cells and edema, findings again mimicking those from naturally acquired infections [2]. Nevertheless, high challenge doses were not markedly toxic to the host. A classic-type enterotoxin, such as has been described for *C. jejuni*, would not alone explain these clinical features of illness. It is further important to note that two strains that did not produce enterotoxins by the methods of Klipstein et al. [10] were fully capable of causing illness in humans. This study did not resolve whether tissue invasion or cytotoxin was the major pathogenetic determinant [27].

On the basis of higher attack rates at equal doses and a greater number and volume of stools produced, the strain derived from an outbreak, 81-176, appeared to be more pathogenic than the sporadic strain, A3249. The similar outcome of both infections in the RITARD model [17] suggests either that these two organisms are of roughly similar virulence or that the RITARD model is relatively insensitive to strain differences. The behavior of strain A3249 is notable in that in vitro this organism switched with high frequency to an aflegellated form, but after passage through volunteers, only flagellated forms were isolated. Nevertheless, the heat-stable lipopolysaccharide antigen did not change. This switching is exactly as has been reported after in vitro plating and passage through rabbits [28] and suggests that in vivo passage selects for flagellated forms. The presence of flagellae may be a virulence factor for *C. jejuni*, as it is for *Vibrio cholerae* [29].

Although bacteremia and other extraintestinal manifestations of *C. jejuni* infections are well described in compromised hosts, they are reported to occur in immunocompetent hosts as well [19]. The absence of bacteremia detected in our studies, despite very extensive surveillance, suggests that bacteremia is a very uncommon event in immunocompetent hosts. The rapidity of clearance of the organisms from stools after erythromycin treatment mimics observations after natural infection [30].

Our studies clearly indicate that short-term homologous immunity to *C. jejuni* exists. That immunity may occur under natural settings is supported by the lack of illness among chronic drinkers of raw milk who are exposed to an epidemic strain [31]. Infection with *C. jejuni* is hyperendemic in Bangladesh and other developing countries [3], and acquisition of immunity may best explain the age-related decrease in the case-to-infection ratio [5]. *C. jejuni*-specific serum antibodies are present at significantly higher levels in children in developing countries than in the United States [23, 32].

Serological studies confirm that the group antigen is recognized by persons infected with two *C. jejuni* strains and that antibody responses after experimental challenge mimic those occurring after naturally acquired infection [22, 33]. The conditions of our volunteer trials permitted a precise delineation of the temporal sequence of the serological response and indicated that for all three immunoglobulin classes, the peak response occurred at day 11 after challenge and levels gradually declined toward the baseline by day 28. That infected persons who developed illness showed greater serological responses than did those who became infected but remained well suggests that the severity of the clinical consequence of infection may be a determinant of host humoral response and parallels the observations made for other enteric pathogens, including *V. cholerae* [34] amd *Shigella* [35]. Whether the serum antibody responses observed in infected persons are protective themselves or merely reflect other protective mechanisms could not be answered by this study.

In conclusion, both strains of *C. jejuni* studied in volunteers were able to establish infection, first in the small intestine and later in the colon, and to cause diarrheal illnesses with many of the features of naturally acquired infection. These illnesses were

more severe with one strain than the other and ranged from watery diarrhea to dysentery with blood and white blood cells in the stool; infection was an immunizing event. The clinical characteristics, as well as the rectal biopsy findings, suggest that an acute inflammatory response is the hallmark of these infections. Additional work is needed to further characterize the exact pathogenic mechanisms causing the inflammation.

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