INFECTION AND IMMUNITY, Oct. 1984, p. 64–67
0019-9567/84/100064-04$02.00/0
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Vol. 46, No. 1

Adoptive Transfer of Immune Enhancement of Experimental Ulcerative Colitis

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Received 4 May 1984/Accepted 3 July 1984

Previous experiments with the carrageenan model for ulcerative colitis have shown that the inflammatory response in guinea pigs can be enhanced by immunization with and subsequent feeding of *Bacteroides vulgatus* to experimental animals. The present studies showed that only certain strains of *B. vulgatus* are capable of provoking immune enhancement of ulcerative colitis. Animals were fed carrageenan and various strains of viable *B. vulgatus* after immunization with a strain of *B. vulgatus* isolated from a guinea pig with experimentally induced colitis. Histological comparison of immune and nonimmune groups revealed that immune animals which received *B. vulgatus* from a patient with inflammatory bowel disease had a significantly ($P < 0.025$) greater number of histopathological lesions at 21 days than did nonimmune animals. Immune animals receiving *B. vulgatus* isolated from a clinically normal source did not show any significant difference in disease status when compared to nonimmune animals. Additional experiments showed that adoptive transfer of spleen cells from animals immunized with *B. vulgatus* to nonimmune recipient animals is effective in transferring the immune enhancement demonstrated in actively immunized animals. Animals which received immune spleen cells with concurrent feeding of *B. vulgatus* showed a significant ($P < 0.005$) increase in inflammation over control groups, in the absence of high titers of circulating antibody. These experiments indicate that *B. vulgatus* strain-specific factors are important to immune enhancement of experimental disease and also suggest an involvement of the cell-mediated immune system in this model.

Experimental ulcerative colitis can be induced in guinea pigs by the oral administration of a red seaweed extract, carrageenan, as first described by Watt and Marcus in 1969 (12, 13). Administration of a 5% (wt/vol) solution of carrageenan via the drinking water to guinea pigs resulted in the development of ulcerations in the large intestine and cecum within 30 days. Histological changes similar to those described in humans with ulcerative colitis were present in animals on this regimen. It has been reported that germfree guinea pigs given carrageenan do not develop cecal or large bowel ulcerations until associated with pools of bacteria in which *Bacteroides vulgatus* is a common factor (7). Association of germfree guinea pigs with *B. vulgatus*, with or without carrageenan treatment, resulted in the development of intestinal ulcerations. Previous studies have been designed to define the role of *B. vulgatus* in experimental disease, in part, by determining whether immunization with *B. vulgatus* before carrageenan treatment altered the experimental disease process. In animals immunized with *B. vulgatus*, evidence of more rapid development of disease was noted, as well as some antigenic specificity for *B. vulgatus*. In contrast, immunization with a phenotypically similar organism, *Bacteroides fragilis*, did not provoke a more pronounced inflammatory response after 21 days of carrageenan treatment. Gross observations at necropsy indicated that feeding *B. vulgatus* to *B. vulgatus*-immune animals, with or without carrageenan treatment, resulted in significantly more severe cecal inflammation than in nonimmune groups (6) at 21 days. It was also noted that carrageenan treatment of immunized animals without viable *B. vulgatus* feeding, did not significantly alter the results as compared with carrageenan treatment in nonimmune animals. The present study extends the observations of the role of the immune response to *B. vulgatus* in the carrageenan model system. In addition, isolates of the same species from different sources were evaluated in an effort to determine whether bacterial strain specificity is an important factor for immune enhancement.

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MATERIALS AND METHODS

***B. vulgatus* strains.** *B. vulgatus* TUSVM 40G2-33, originally isolated from a guinea pig with cecal ulcerations, was obtained from the stock culture collection of the Infectious Disease Research Laboratory, Tufts University School of Veterinary Medicine, Boston, Mass. *B. vulgatus* strains 12-5 and 10-9 were isolated from the feces of healthy humans without ulcerative colitis, and *B. vulgatus* 20-15 was isolated from the rectal washings of a patient with ulcerative colitis. Stool samples from the healthy individuals were serially diluted in phosphate-buffered saline from 10^{-2} to 10^{-9}. Aliquots (0.1 ml) of these dilutions were plated onto brucella-based blood agar with hemin and vitamin K_1 (BMB) and laked blood agar containing kanamycin and vancomycin to select for gram-negative bacteria. The plates were incubated for 48 h in an anaerobic chamber at 37°C (Labline Instruments, Melrose Park, Ill.), and the various colony types were subcultured onto BMB for isolation. Isolates were gram-stained and checked for aerotolerance on chocolate agar. All gram-negative anaerobic bacteria were identified by the Anastat System (Scott Laboratories, Fiskeville, R.I.). The *B. vulgatus* strains isolated from rectal washings of the patients with ulcerative colitis were processed in the same manner.

Animals. Animals used for active immunization and adoptive transfer experiments were male Hartley guinea pigs (CRL:CHA/BR; Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Animals used for active immunization

weighed 600 to 650 g, whereas those used for adoptive transfer weighed 300 to 350 g. During experimentation, animals were housed two to four per cage in stainless steel cages and received food (vitamin C-enriched Ralston Purina, St. Louis, Mo.) and carrageenan ad libitum. Experimental colitis was induced by the administration of a 5% (wt/vol) solution of sterile carrageenan as the sole source of oral fluids.

Challenge with *B. vulgatus*. Animals challenged with viable *B. vulgatus* received 0.5 ml of a 24-h culture grown in brain heart infusion broth once daily for 21 days by oral intubation. This inoculum was calculated to contain 10^9 CFU/ml based on preliminary experiments.

Immunization procedure. Animals were immunized with a washed, Formalin-killed bacterial cell suspension adjusted to contain the equivalent of 2×10^8 CFU/ml. Guinea pigs were injected once weekly for 3 weeks in each footpad with 0.05 ml of the bacterial suspension in 50% (vol/vol) Freund incomplete adjuvant (Difco Laboratories, St. Louis, Mo.) and twice weekly subcutaneously with a 0.1-ml aliquot of the washed cell suspension in phosphate-buffered saline (10^8 CFU/ml). All animals were boosted with a 0.1-ml subcutaneous injection of the cell suspension after a 1-week rest period.

Spleen cell preparation. Spleens of immune and nonimmune animals were aseptically excised, and the fat and connective tissue were cut away. The spleens were gently minced through a stainless steel screen into L15 media (Flow Laboratories, McLean, Va.) with 10% fetal calf serum. The cells were suspended in fresh media without fetal calf serum, electronically counted with a Coulter FN cell counter, and diluted to the appropriate concentration. Viable cell density was measured by trypan blue exclusion. Animals were bled and checked for serum agglutinating antibody titer before experimentation and at the time of necropsy by a bacterial agglutination procedure (11).

Evaluation of animals. Animals were sacrificed when they became moribund or at the end of 21 days of experimental treatment. During necropsy the peritoneal cavity was exposed, and the gross appearance of the large intestine and cecum was recorded. The animals were bled, and the serum was frozen for agglutinating antibody titer measurement. Tissue was removed from the same area of the cecum, rectum, and colon of each animal by a modification of the Swiss roll technique (4). The tissues were fixed in 10% neutral-buffered Formalin and processed by standard techniques for microscopic evaluation. Each tissue was scored from 0 to 4 on the basis of the severity of pathological changes. Crypt abscess formation, epithelial thinning, loss of crypts, polymorphonuclear cell infiltration, and obvious ulcerations were the characteristics evaluated as previously described (6). The total score was determined by adding the scores for each tissue. Since three tissues per animal were evaluated, a maximum total score of 12 could be achieved. The scores for each animal in a particular group were used to determine the mean histological score. Each tissue was evaluated independently by two investigators in a blind fashion.

Experimental design. Strain specificity and cross-reactivity experiments. Three groups of animals were placed on carrageenan treatment subsequent to immunization with *B. vulgatus* TUSVM 40G2-33 (6). Two of these groups were fed viable *B. vulgatus* daily, with one group receiving *B. vulgatus* 20-15 isolated from a human patient with inflammatory bowel disease and the other group receiving *B. vulgatus* 12-5 isolated from a healthy individual. The third immune group received only carrageenan. An additional group of nonimmune animals were also included as carrageenan recipients. To test for cross-reactivity between the various strains of *B. vulgatus*, four guinea pigs were immunized with *B. vulgatus*, two were immunized with *B. vulgatus* 10-9, and two were immunized with *B. vulgatus* 20-15. At the end of the 4-week immunization procedure, the animals were bled and sacrificed. Sera were tested for antibody titer by the bacterial agglutination procedure with strains 10-9, 20-15, and TUSVM 40G2-33 as antigens.

Adoptive transfer experiments. Four groups of animals received 10^7 spleen cells by intraperitoneal injection 1 day before the start of carrageenan treatment. Two groups received spleen cells from animals that had been immunized with *B. vulgatus* 20-15, and two groups received spleen cells from naive animals. Viable *B. vulgatus* 20-15 was fed to two of the groups as described below.

RESULTS

Strain specificity experiments. Four groups of animals on carrageenan treatment for 21 days were used in this experiment, three immune and one nonimmune group. The control group used for comparison was comprised of nonimmune guinea pigs receiving only carrageenan to induce experimental colitis (Table 1). The immune group receiving only carrageenan showed a lower histological score than did the nonimmune group, indicating that immunization with *B. vulgatus* without challenge by oral feeding of *B. vulgatus* does not enhance the disease process. The mean histological score in the group challenged with viable *B. vulgatus* 20-15 isolated from a patient with inflammatory bowel disease was significantly higher than that in other groups. The group challenged with *B. vulgatus* 12-15 isolated from the stool of a healthy individual showed the lowest histological score among all groups. Very little serological cross-reactivity between *B. vulgatus* strains was detected based on assays in which guinea pig immune sera and heterologous strains of *B. vulgatus* were used (Table 2).

Effect of passive transfer. An attempt to determine whether immune enhancement could be passively transferred to nonimmune animals was made by using spleen cells from actively immunized animals. A comparison of the mean histological scores for groups which received spleen cells from naive animals were similar regardless of oral *B. vulgatus* challenge. Groups receiving spleen cells from animals immunized with *B. vulgatus* 20-15 showed an increase in the mean histological score compared to groups receiving spleen cells from naive animals. Immune spleen cell recipients

TABLE 1. Strain specificity of the immune response in experimental colitis

Experimental condition[a]	Immune status	Histological score (mean ± SEM)[b]
Control (no challenge) (5)[c]	Nonimmune	6.20 ± 0.984
No challenge (4)	Active *B. vulgatus* TUSVM 40G2-33	4.50 ± 0.500
Challenge (*B. vulgatus* 12-5) (4)	Active *B. vulgatus* TUSVM 40G2-33	4.33 ± 0.877
Challenge (*B. vulgatus* 20-15) (8)	Active *B. vulgatus* TUSVM 40G2-33	8.85 ± 0.668 ($P < 0.025$)[d]

[a] All animals were given carrageenan for 21 days.
[b] Mean group score for all tissues. 0, No evidence of disease; 12, ulceration seen in all tissues.
[c] Numbers in parenthesis are the number of animals in each group.
[d] *t*-test versus nonimmune carrageenan recipients.

TABLE 2. Cross-reactivity of *B. vulgatus* isolates for agglutinating antibody

Animal no.	Immunizing *B. vulgatus* strain	Antigen		
		B. vulgatus 10-9	*B. vulgatus* 20-15	*B. vulgatus* 40G2-33
1	10-9	1:4,096	—[a]	—
2	10-9	1:770	1:8	1:4
3	20-15	1:12	1:4,069	1:8
4	20-15	1:8	1:384	1:4
5	40G2-33	1:4	1:6	1:4,096
6	40G2-33	1:8	1:8	1:1,024

[a] —, No detectable response.

challenged with *B. vulgatus* 20-15 showed a significantly higher histological score than the comparable naive spleen cell recipients. Circulating antibody was absent or detected at a low titer in immune spleen cell recipients.

DISCUSSION

Bacterial involvement in the experimental model of ulcerative colitis has been studied extensively (5, 7–9). Data from numerous experiments suggest that gram-negative anaerobic bacteria play an important role in the experimental disease process. Previous studies in germfree animals indicated that animals fed carrageenan did not develop the experimental disease until associated with a microflora which included *B. vulgatus* (7). Animals which were monoassociated with *B. vulgatus* were shown to develop ulcerations in the presence or absence of carrageenan treatment, although the disease was more pronounced when carrageenan was part of the experimental regimen. Although the role of carrageenan in this experimental disease is not clear, recent evidence suggests that carrageenan may have some unique properties (unpublished data) which include assisting *B. vulgatus* antigens in crossing the mucosal barrier. The immune enhancement of the experimental disease caused by *B. vulgatus* is an interesting feature of this model system (6). This bacterium is part of the normal microflora in many animal species as well as in humans, and its potential role in the experimental disease process does not appear to be as a traditional enteric pathogen. Immunological involvement of one or more components of the gut microflora has often been suggested as a possible etiological mechanism in ulcerative colitis. Monteiro et al. (3) considered the possibility of antibody-mediated hypersensitivity playing a role in the human disease. These investigators found antibodies in the colonic mucosal tissues from patients with colitis as well as from patients with other inflammatory bowel diseases which reacted with obligate anaerobes isolated from the patients with colitis. These investigators also suggested that the mucosal antibody resulted from antigenic stimulation by anaerobic bacteria present in the gut lumen. The data indicated that the mucosal antibody belonged to the immunoglobulin G class, but it was not found in serum. Other studies such as that of Bendixen (1) have also reported evidence of a cell-mediated immune response directed against the bacterial residents of the large bowel. Recent experiments have studied the effect of immunization with *B. vulgatus* on the course of the carrageenan-induced disease in guinea pigs (6). The data indicate that ulcerations develop more rapidly and more severe lesions are present at 21 days after immunization with *B. vulgatus* (6). The results from the present experiments support these earlier experiments, although the role of serum antibody as a mediator is suspect since adoptive transfer provokes disease in some animals in the absence of detectable antibody. In addition, immunization with strains of *B. vulgatus* isolated from patients with ulcerative colitis and from other sources appear to vary in their effects on the development of the experimental disease. *B. vulgatus* isolated from a healthy individual did not exacerbate ulcer development when fed to animals immunized with *B. vulgatus* isolated from a guinea pig with carrageenan-induced colitis. The opposite was true when a strain of *B. vulgatus* from a patient with inflammatory bowel disease was used in this model system. These data suggest a possible strain specificity among *B. vulgatus* isolates or that *B. vulgatus* from the patient with ulcerative colitis produced some factor not found in the other strains. Earlier experiments with this animal model have shown that immunization and association of animals with *B. fragilis* does not result in any change in the experimental disease (6). *B. fragilis* is phenotypically similar to *B. vulgatus*, but serogrouping data and DNA homology studies have shown it to be a distinct species and antigenically different from *B. vulgatus* (2). The results noted in these experiments suggest that there may be different serogroups among *B. vulgatus* strains, just as there are among strains of *B. fragilis*.

The immune enhancement noted with active immunization was also produced in recipients of spleen cells from actively immunized animals. These data suggest the involvement of the cell-mediated immune system. Spleen cell recipients had little or no detectable antibody titers to the homologous *B. vulgatus* strain, yet still exhibited the same disease enhancement seen in actively immunized animals. This observation is consistent with the fact that circulating antibody to the challenge strain in actively immunized animals was often low, but animals still developed disease. That adoptive transfer of immune spleen cells resulted in immune enhancement also suggests that the strain specificity observed is not due to some factor produced only by *B. vulgatus* in situ. These findings are supported by the studies of other investigators who have (10) developed a nonantibody-mediated model of inflammatory bowel disease in rabbits. Animals skin sensitized with dinitrochlorobenzene and challenged by instillation of dinitrochlorobenzene into the colon develop colonic ulcerations. Repeated instillation results in chronic ulcerations and a pronounced delayed hypersensitivity skin reactivity. The lesions found were only present as long as dinitrochlorobenzene stimulation was present. These investigators concluded that as long as a cellular immune response occurs in the wall of the colon and exogenous antigen is available, the lesions of this experimental inflammatory bowel disease persist. A bacterial antigen could be available to the colonic mucosa at all times and may explain the persistence and recurrence of lesions in ulcerative colitis.

In this study we have seen an enhancement of disease through an immune response to *B. vulgatus* which does not appear to correlate with circulating antibody. The possibility that the assay used to assess antibody was not sensitive enough or that the cell-mediated system is involved is being evaluated. Earlier experiments have strongly implicated *B. vulgatus*, an obligately anaerobic bacterium, as having an important role in the experimental disease. These data suggest that experimental colitis may be the result of a cell-mediated reaction due to bacterial antigen(s) in the large bowel. The strain-specific effects of *B. vulgatus* are currently being evaluated with additional strains. Phenotypic characterization of the *B. vulgatus* strains used in these studies show no obvious metabolic differences. The presence of plasmids, unusual enzymatic activities, and possible cytopathic effects of the various strains in vitro are currently being evaluated.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant R01 AM26452 from the National Institute for Arthritis, Diabetes, Digestive and Kidney Diseases.

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