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Inflammatory Bowel Disease Induced by Combined Bacterial Immunization and Oral Carrageenan in Guinea Pigs

Model Development, Histopathology, and Effects of Sulfasalazine

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A model of experimentally induced inflammatory bowel disease (IBD) featuring colitis, originally devised by Onderdonk and co-workers in guinea pigs, was modified to establish the optimal conditions for ulcer development. Upon varying the time of subcutaneous immunization with Bacteroides vulgatus *and concomitant oral administration of acid-degraded* iota-carrageenan *and viable* B. vulgatus, *it was found that the optimal times of administering these agents were one to two weeks and five to six days, respectively. Light microscopy of the colon and cecum of the guinea pigs given the optimized treatment for ulcer induction revealed pronounced edema, inflammation, and lesions of the mucosa. Transmission electron microscopy of the mucosa from these animals showed the presence of large numbers of leukocytes in the subepithelial region, the majority being polymorphonuclear neutrophils which possessed large electron-dense granules or rods. Oral administration of 300 mg/kg/day sulfasalazine (salicylazosulfapyridine) for 14 days to guinea pigs given the optimized treatment for ulcer induction failed to reduce the numbers of ulcers or the histopathology gradings and fine structural changes of the mucosal inflammatory changes, but did reduce the symptoms of diarrhea.*

KEY WORDS: inflammatory; intestinal tract; colitis; sulfasalazine; ulcers.

Ulcerative colitis and related inflammatory bowel diseases (IBDs) present a major therapeutic challenge today inasmuch as current drugs fail to exert long-term control or reversal of the disease process and they also have appreciable adverse effects (1, 2). The search for more effective agents depends on the suitability of models developed in laboratory animals to reproduce the pathology of IBDs as seen in man so as to serve as a screen for new drugs and to study their modes of action. The suitability of these animal models depends upon recognizing the nature of inflammatory response characteristic for the disease in man. Unfortunately, in practice, the distinction between the histopathology of the two major IBDs, ulcerative

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colitis and Crohn's disease, is often unclear because these types often present with a spectrum of histopathology (3, 4).

Generally, ulcerative colitis is regarded as a disease of the colon beginning in the rectum (3, 4) with histologic features of a classic Arthus-type reaction and characterized by an extensive acute inflammatory infiltrate comprising polymorphonuclear neutrophil leukocytes (PMNs), macrophages, eosinophils, and plasma cells with accompanying edema, vasodilatation, and diapedesis (3, 4). Classically, as with all Arthus type reactions, it is immunologically initiated and sustained by enhanced complement components especially C3a, immunoglobulin G (IgG), and circulating IgG immune complexes (5–7) whose role in pathoetiology of the disease is unclear (8). This hyperimmune state implies the possibility that ulcerative colitis is a type-III hypersensitivity reaction with essentially B-cell activation.

Of the various models of ulcerative colitis that have been developed (reviewed extensively in references 9–11), those involving "B-cell models" would, therefore, seem to have somewhat greater relevance to ulcerative colitis in man, although "T-cell models" can elicit acute IBD conditions (10). Of particular relevance in the choice of animal models is that, once initiated, the disease should be relatively self-sustaining (10). While approaching this requirement, the B-cell models initially developed by Kirsner and his colleagues (12, 13) and modified extensively by others (9–16) may be representative of the human colitis. The procedure essentially involves sensitization to an antigen by systemic administration of the antigen followed by local injection of the antigen into the intestine to elicit mucosal inflammation. One of the versions of this model developed by Onderdonk and his colleagues employs *Bacteroides vulgatus* as the immunizing antigen (14, 16). They found that the immunizing procedure in guinea pigs enhanced the ulceration induced by oral administration of carrageenan, an inflammagen that alone elicits ulcerative colitis in this species (17) but whose response has been notoriously variable and unreliable (18). While the *Bacteroides* with carrageenan dosing model offers considerable promise as a routine laboratory model, important details are required regarding the optimization of the immunization procedure and detailed histopathology to define the nature of the induced colitis in comparison with that in man. We, therefore, decided to perform studies on these aspects so as to provide a more practical and representative animal model of IBD in man and to examine the effects of a standard antiulcer drug, sulfasalazine (=SASP, salicylazasulfapyridine), in this model.

MATERIALS AND METHODS

Induction of Intestinal Inflammation. Two studies were performed using male Hartley guinea pigs (approximately 400–500 g initial body weight). In the first study, three groups of 15 animals each were given two subcutaneous injections per week of 0.1 ml formalin-killed *Bacteroides vulgatus* (prepared as described later) in phosphate-buffered saline with a further injection of the bacteria suspended in Freund's complete adjuvant, once weekly, for periods of one, two, and three weeks, respectively. A booster injection of the same quantity of killed *B. vulgatus* suspended in phosphate-buffered saline was given subcutaneously one week following the last injection. Following the final immunization, each of three animals received 5% w/v acid-degraded iota-carrageenan (prepared as described later) and viable *B. vulgatus* (2×10^8 CFU) *per os* daily for 0, 4, 7, 10, and 14 days. Body weights and the appearance of stools and animals' physical appearance were noted daily. The animals were killed by CO_2 asphyxiation at 0, 4, 7, 10, and 14 days, respectively. Gross pathological observations of the intestinal tract were recorded and sections of the ulcerated and nonulcerated cecum, colon, and rectum were taken for light microscopy. Antibody levels in the sera were determined at termination in all the groups above as a means of assessing the immune reaction.

In the second study, the effects of sulfasalazine treatment were investigated in animals that were immunized for the two-week period using the procedures described above with the degraded carrageenan and viable *B. vulgatus* being given orally each day for the last seven days of this period. The animals were left untreated for the remaining four days before the commencement of drug treatment to avoid osmotic effects of carrageenan interfering with drug absorption. Groups of seven animals each were then dosed orally with a single dose of 300 mg/kg/day sulfasalazine in 5 ml of 0.25% w/v aqueous methylcellulose for 10 days, respectively. Control animals received an equivalent volume of the methylcellulose solution alone. The body weights and conditions of the animals were recorded and gross and microscopic pathology determined as in the first study.

Preparation of Degraded Carrageenan. Sodium iota-carrageenan, 50 g (type X-5852 supplied by the Copenhagen Pectin Factory, Lille Skensved, Denmark DK-4623) was added to 900 ml distilled H_2O and 25 ml 1 N HCl and heated for 3½ hr at 70° C. Sucrose (25 g) then was added to improve the taste, and the solution was neutralized to pH 7.0 by addition of approximately 1.5 ml 1 N NaOH. The final volume was adjusted to 1 liter with H_2O.

Preparation of *B. vulgatus*. *B. vulgatus* strain TISVM 40G2-22, generously donated by Dr. A. Onderdonk, was grown in brain HEMPT infusion broth in 4 ml Nunc cryotubes gassed with N_2. The bacteria were prepared in sterile phosphate-buffered saline mixture for injection.

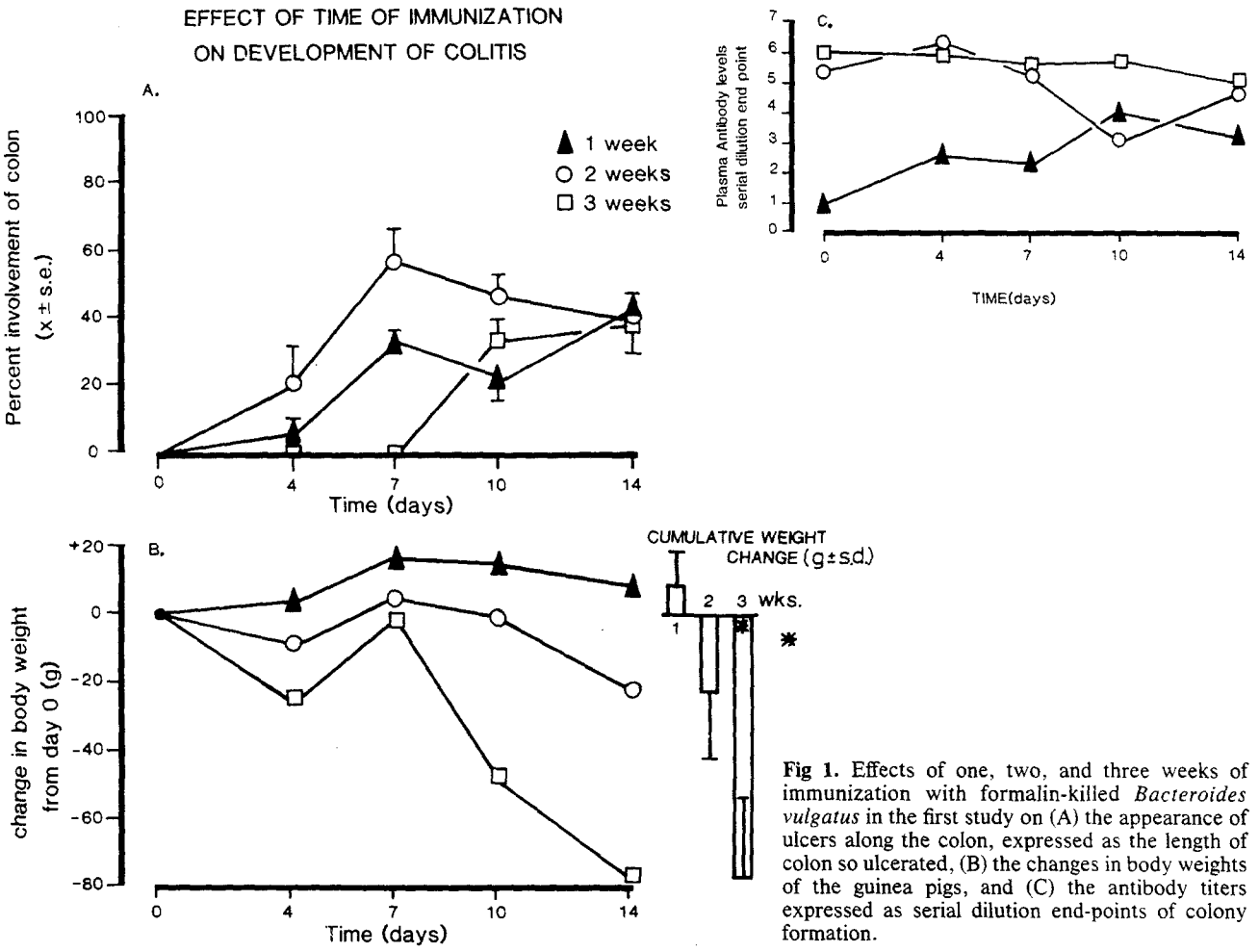

Fig 1. Effects of one, two, and three weeks of immunization with formalin-killed *Bacteroides vulgatus* in the first study on (A) the appearance of ulcers along the colon, expressed as the length of colon so ulcerated, (B) the changes in body weights of the guinea pigs, and (C) the antibody titers expressed as serial dilution end-points of colony formation.

The bacteria used for immunization were killed by addition of 10% formalin and placed at 4° C overnight. The cells then were centrifuged and washed with sterile phosphate-buffered saline.

Light Microscopy. Selected areas of the mucosa from the large intestine where ulcers or lesions were visible, as well as nonulcerated areas of control or SASP-treated animals, were fixed in 4% formaldehyde in phosphate-buffered saline, embedded in paraffin, and sections stained with hematoxylin and eosin. The assessment of pathological changes was made on a blinded basis by a trained veterinary pathologist (T.G. Hodge, DVM) who was unaware of the treatment schedule and independently also by K.D.R.

Scanning Electron Microscopy (SEM). Selected ulcerated and nonulcerated regions of the cecum, colon, and rectum from guinea pigs in the second study, ie, from both the controls and those dosed with SASP were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for one to five days before critical point drying, mounting, and sputter-coating with gold. Sections of regions from the large intestine of a separate group of four guinea pigs that had not received any treatments were similarly selected and prepared for comparison as normal control material. Specimens were examined by a JOEL JEM 100C scanning electron microscope at an accelerating voltage of 100 kV.

Transmission Electron Microscopy (TEM). Tissues were selected for TEM from animals treated as for the SEM studies. These tissues were fixed in 2.5% glutaraldehyde and 2% formaldehyde prepared in 0.1 M sodium phosphate buffer, pH 7.4, for 1–6 hr, postfixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.4. The sections were subsequently dehydrated through a graded series of aqueous ethanol solutions and 1,2-epoxypropane prior to embedding in Epon. Thin sections were selected following examination of toluidine blue-stained thick sections, and the former stained with lead citrate (19) and uranyl acetate (20) prior to examination under a Phillips 300 EM at an accelerating voltage of 100 kV.

RESULTS

Development of Intestinal Inflammation. The data (Figure 1A–C; Table 1) show that a progressive increase occurred with time in the extent of ulceration in the colon (Figure 1A) concomitant with a

TABLE 1. GROSS PATHOLOGICAL OBSERVATIONS IN BACTERIAL IMMUNIZATION/CARRAGEENAN MODEL OF COLITIS

Organs/pathology	Average scores* after time immunized (week) 1	2	3
Cecum			
Diffuse mucosal edema and reddening	3	3–4	4
Enlarged submucosal lymphoid nodules	3	4	4
Colon			
Diffuse mucosal edema and reddening	2–3	2–3	2–3
Multifocal transverse mucosal fissures	1–3	3	2–3
Rectum			
Mucosal edema and reddening	1–2	2	2
Focal mucosal ulcers and hemorrhage	2	2–3	2–4

*Scoring: 0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe.

decline in body weight (Figure 1B), which increased with the time of immunization, the effects of which were confirmed by increase in antibody titers (Figure 1C). The percentage of animals with diarrhea also increased with time, but this was fully manifest in all animals by five to six days of carrageenan treatment (Figure 2). The diarrhea appeared to develop in advance of chronic ulceration and inflammation in the large intestine.

The gross pathological changes observed (Table 1) included pronounced edema, inflammation, and

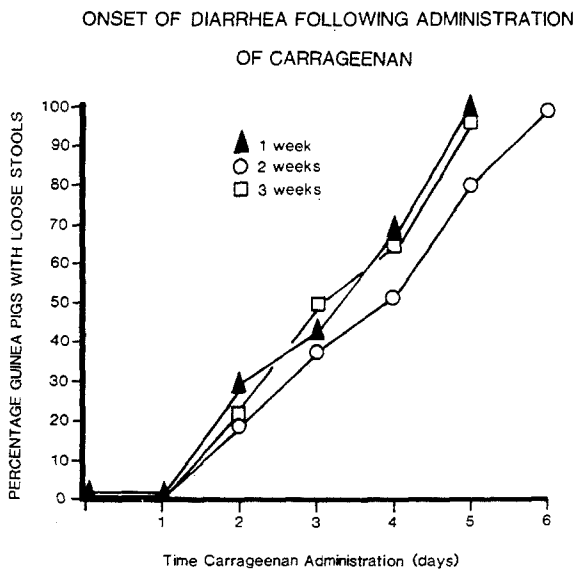

Fig 2. Time course of the development of diarrhea, expressed as the percentage of animals with loose stools, during the first six days of oral administration of carrageenan with live *Bacteroides vulgatus* in the first study. No differences were evident between the groups of animals treated for one, two, and three weeks with the formalin-killed bacteria.

TABLE 2. EFFECT OF SULFASALAZINE ON RANGE OF SCORING* GROSS PATHOLOGICAL OBSERVATIONS IN GUINEA PIGS WITH COLITIS

	Control	SASP (300 mg/kg/day)
Cecum		
Diffuse mucosal edema and reddening	1–4	2–4
Enlarged submucosal lymphoid nodules	1–4	1–3
Colon		
Diffuse mucosal edema and reddening	2–4	2–3
Multifocal transverse mucosal fissurelike lesions	2–4	1–4
Rectum		
Mucosal edema and reddening	2–4	1–3
Focal mucosal ulcers and hemorrhage	1–4	1–3
Presence of formed stools (%)	0	25

*Scoring: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

mucosal lesions in the colon and cecum one to two weeks after commencement of immunization. Aside from slight mucosal cell injury in the rectum, no further deterioration was evident (Table 1). The ulcers in the colon were uniformly distributed along the entire length of this organ. In their appearance by light microscopy, these ulcers showed extensive cellular infiltration and edema.

It was evident from these studies that, optimally, the establishment of the disease without causing serious debility to the animals, manifest in extensive weight loss, could be achieved by immunization for two weeks. This treatment was, therefore, employed in the subsequent studies comparing the effects of sulfasalazine (300 mg/kg/day). The high doses of this drug failed to produce any appreciable reduction in the percent of animals with mucosal pathology or severity in the large intestine of guinea pigs with colitis, with the possible exception of some slight improvement in the colon and rectum (Table 2). There was also some improvement in the symptoms of diarrhea in the animals in this group (Table 2). However, SASP failed to cause an improvement in the body weight decline observed with progression of the disease, and actually caused a further reduction in the body weights after 10 days of treatment with the drug (Figure 3).

Histopathological assessment of mucosal damage further confirmed the absence of any appreciable improvement in SASP-treated animals. These observations revealed notably severe edema, inflammation, erosions, and hemosiderin

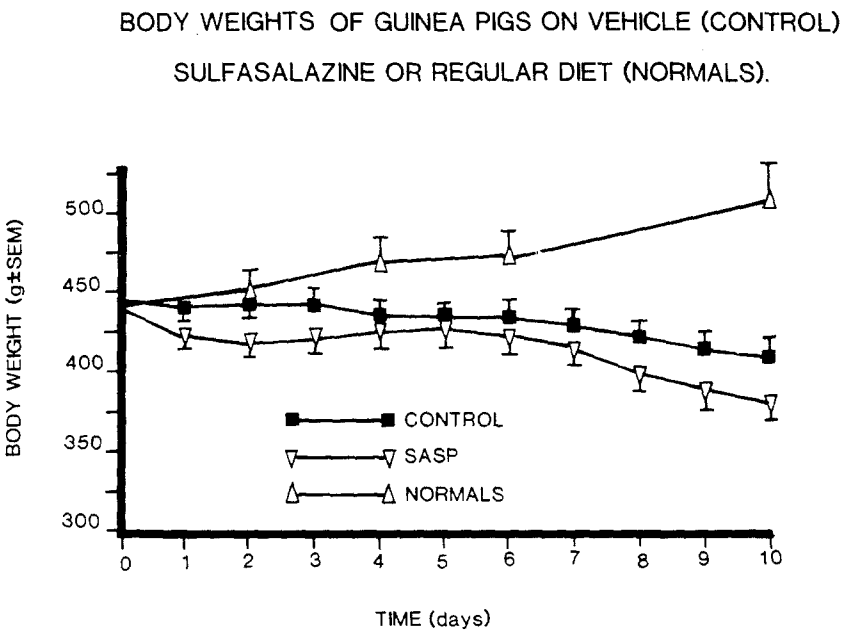

Fig 3. Changes in body weights of guinea pigs with intestinal ulceration established following the two-week immunization schedule in the second study which received either 300 mg/kg/d sulfasalazine or 0.25% methylcellulose vehicle alone (control). The graph shows also the body weight gain for a group of normal untreated control animals for comparison.

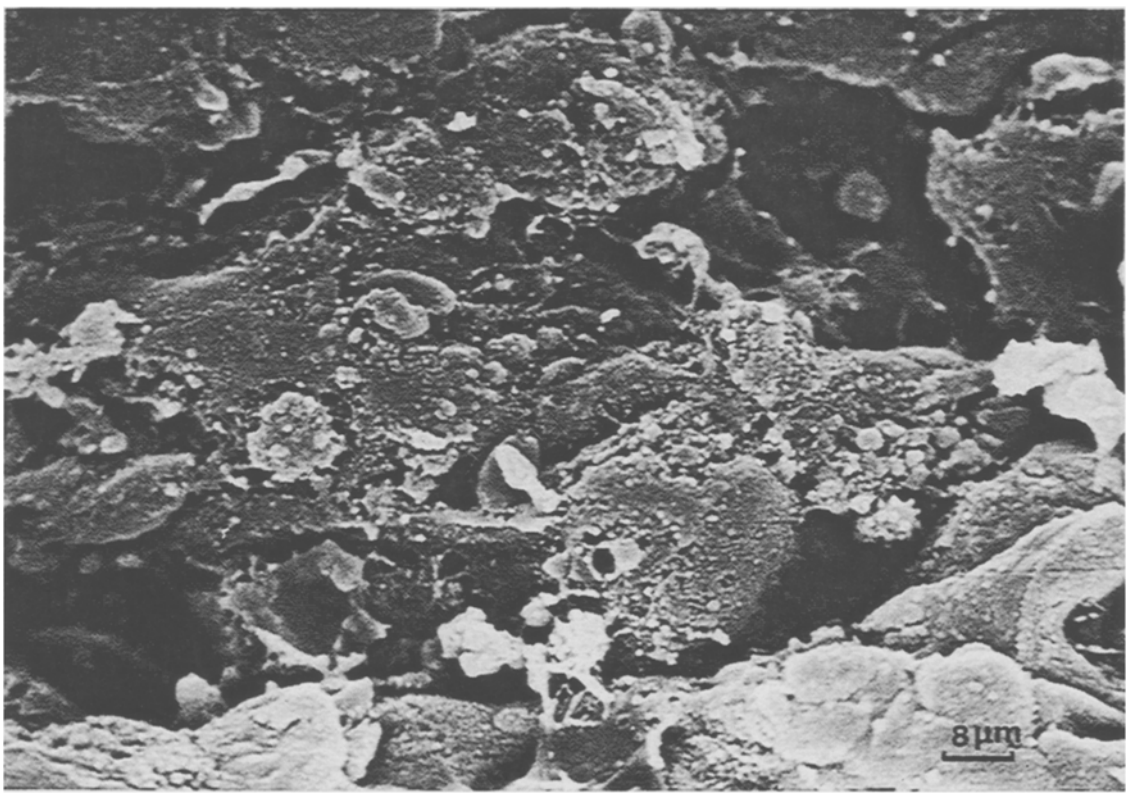

Fig 4. Rectal mucosa from a control animal from the second study in which colitis had been induced, showing erosion and desquamation of the surface mucosa. Clumps of mucus and some eroded mucosal cells and red blood cells are evident.

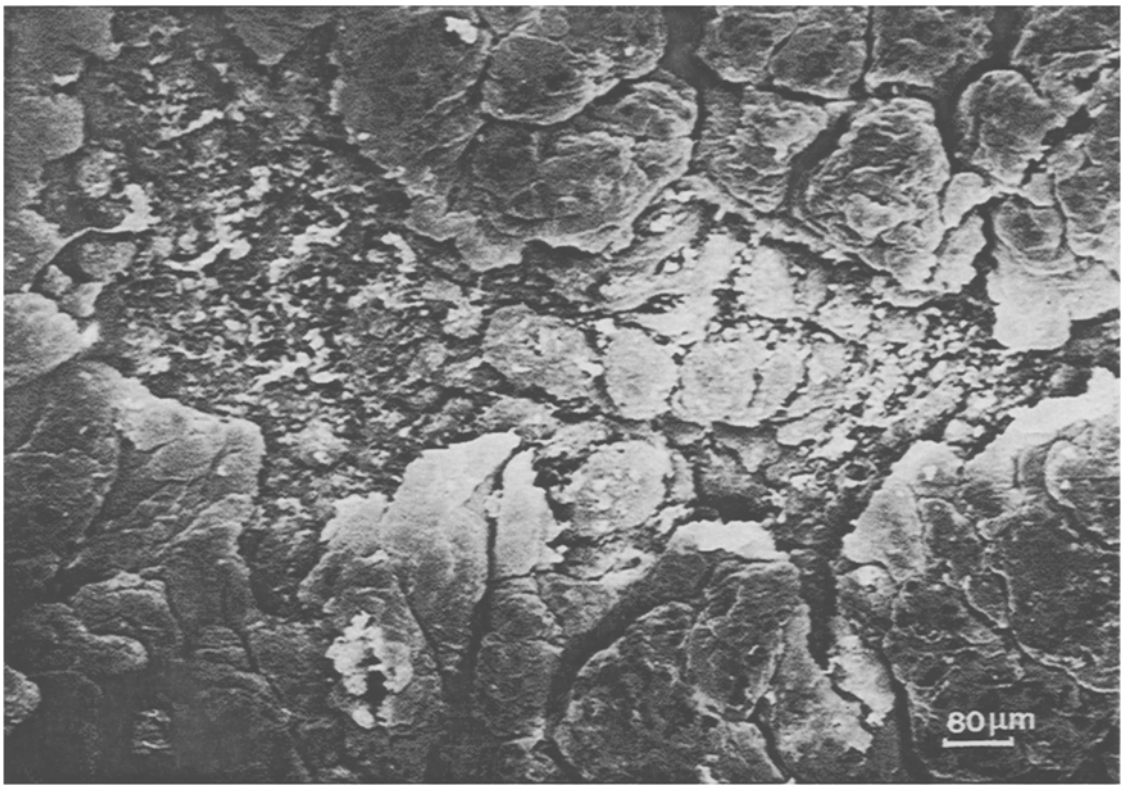

Fig 5. Colonic mucosa from a guinea pig 14 days after induction of colitis, which had received 300 mg/kg/day sulfasalazine for 10 days in the second study. A craterous ulcer is shown centrally and is surrounded by normal although somewhat flattened mucosal cells.

deposits in the cecum of both control and SASP-treated animals.

Scanning (SEM) and Transmission (TEM) Electron Microscopy. SEM of the ulcerated areas of the colonic and rectal mucosa from both control and SASP-treated diseased animals revealed areas of focal erosions of the superficial mucosa (Figure 4) as well as extensive ulcerated craters (Figure 5). The erosions comprised areas of desquamated cells and mucus with cellular debris and blood cells clearly demarcated from the surrounding normal mucosa (Figure 4). The craterous ulcers occupied large areas of the field of view when visualized even by the lowest magnification by SEM (Figure 5).

The erosion and ulcers were evident in both control and SASP-treated animals in whom colitis had been induced and were present in all regions of the colon and rectum. There were no differences apparent in the subjective assessment of mucosa with erosions or, for that matter, with ulcers in animals that received control or SASP treatments.

Under SEM the nonulcerated or noneroded mucosa in all areas of intestinal tract of both control and SASP-treated diseased animals exhibited extensive flattening and distortion of the superficial mucous cells (Figure 5). At high magnification, SEM of the epithelial cells of otherwise undamaged mucosa, especially in the colon, showed a normal appearance as seen in control tissues. Subjectively this appeared about the same in SASP-treated animals.

TEM of the otherwise undamaged mucosa adjacent to lesions or ulcers of animals in whom colitis had been induced revealed relatively normal epithelial cells, except for some reduction, or absence in some cells, or microvilli. There were, however, striking changes in the subepithelial region. In both control and SASP-treated animals there was extensive infiltration of leukocytes in all subepithelial regions of ulcerated and nonulcerated mucosa (Figure 6A and B) and also red blood cells (Figure 6B) accompanied by disrupted or segmented appearance of endothelial cells of capillaries. The most impressive change observed was the large number of leukocytes present with what appeared to be large numbers of irregularly shaped strongly electron-dense granular particles and beadlike rods

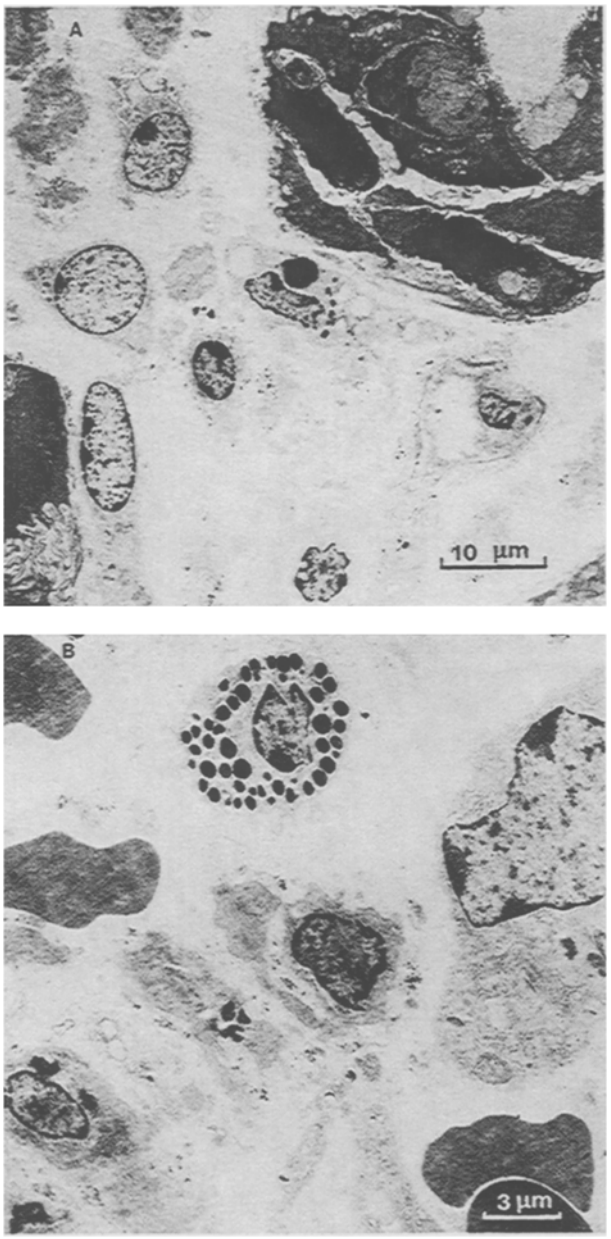

Fig 6A and B. Low-power transmission electron micrographs of the subepithelial region of the rectal mucosa from a control animal 14 days after *Bacteroides*/carrageenan-induced colitis. These sections show a range of leukocytes and red blood cells that have infiltrated this otherwise undamaged region. Identical cellular infiltrates were seen in all regions of the colon of both SASP and control animals. Some eosinophils also were present in the subepithelial zone of the colonic and rectal mucosa, although not shown in these electron micrographs.

(Figure 7A–D). The identity of PMNs as the main cell in which these granules or rods were present was deduced by their size and the presence of the familiar horseshoe nuclei (Figure 7B) asymmetrically located within these cells. Other leukocytes present in appreciable numbers in the subepithelial or interstitial space were plasmacytes (with their large numbers of endoplasmic reticulum) and eosinophils. Extensive swelling of the subepithelial or interstitial region was evident wherein these leukocytes were abundant, reflecting edema. Some damage had occurred to both adjacent epithelial cells and leukocytes in these regions.

DISCUSSION

The results of studies on the time course of the induction of intestinal inflammation featuring ulceration and inflammation of the large intestine show that a simple one to two-week period of immunization is sufficient for the full development of intestinal ulceration, immune response, and associated diarrhea (see Results, Table 1, Figure 1A–C).

The lack of effects of the high dose of 300 mg/kg/day SASP for 10 days on the mucosal pathology in the chronic IBD model developed here indicates that this drug is incapable of affecting severe established chronic disease with more profound immune responses, even though it is obviously capable of antiulcer action in possibly less severe acute animal models, eg, acetic acid and dinitrochlorobenzene (DNCB) induced colitis (9, 10, 22, 23).

The inactivity of SASP on the progress of IBD could be a consequence of the extensive chronic pathology of the disease that may be considered to have a more pronounced immunological involvement, contrasted with more acute inflammatory processes that are known to be inhibited by SASP (21, 23, 24). Thus SASP may only be considered an acute antiinflammatory drug with relatively little activity in chronic states with more severe immunologic involvement, except in reducing symptoms of diarrhea (Table 2).

SASP is known to have a variety of effects on eicosanoid metabolism distinct from that of nonsteroidal antiinflammatory drugs (23, 24) and also affects production of oxygen radical species (25). While the drug has been reported to induce some immunological changes, its the relevance of these effects to the control these aspects of the disease is not clearly established (24). Thus the limited effects of SASP in control of diarrhea might be related to its effects on eicosanoid metabolism (23, 24), whereas the inability of this drug to control or reverse extensive chronic ulceration may be due to its lack of influence on mucosal immunologic and

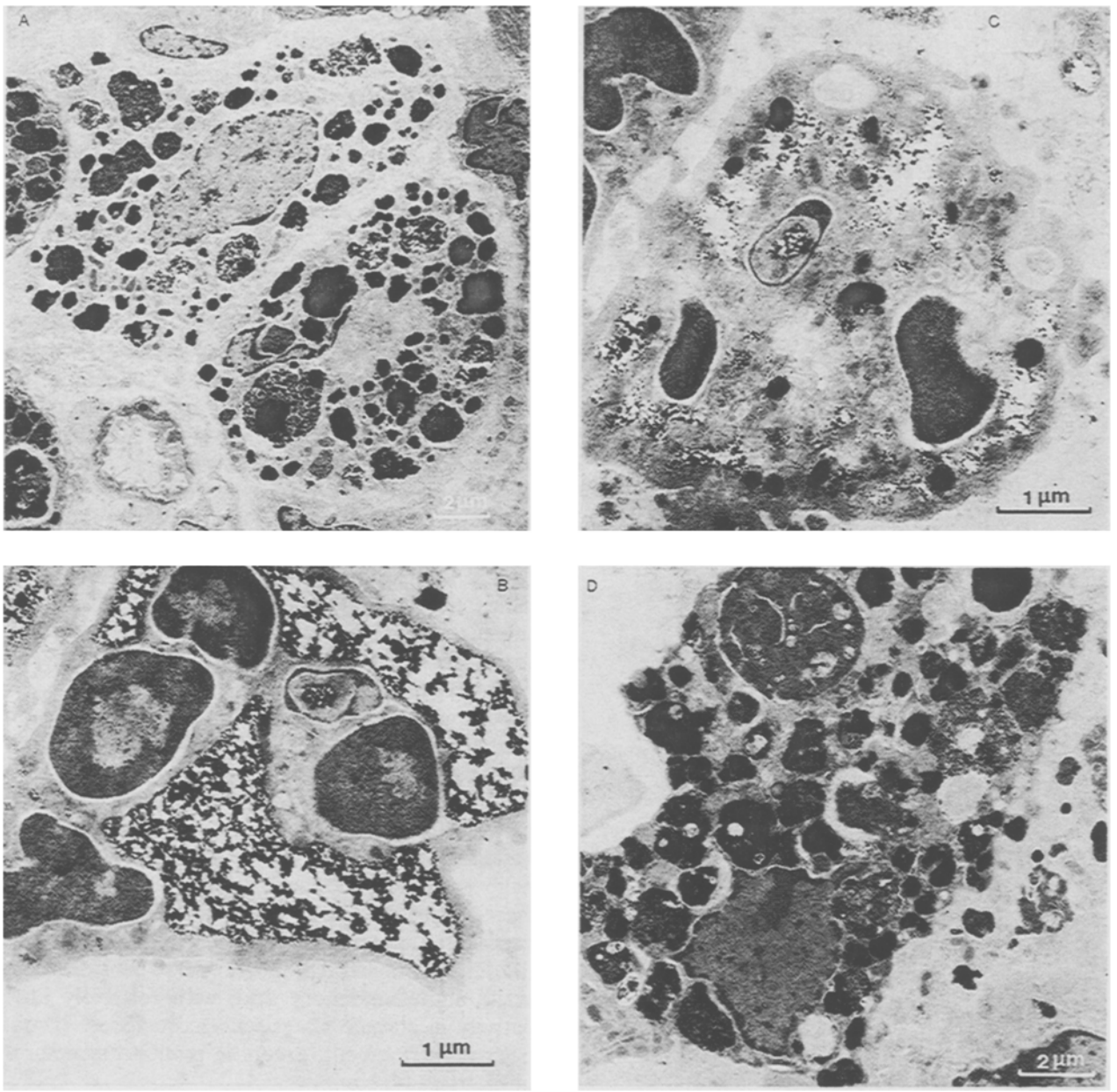

Fig 7. Range of particles or inclusions present in the leukocytes that had infiltrated into the subepithelial region of the colon (A, C, and D) and rectum (B) containing large numbers of electron-dense particles. These appeared as sectional rod or as granular deposits (A and D) and filamentous particles (B and C). These cellular infiltrates were present in both SASP-treated (A and D) and control animals (B and C) with induced colitis.

reparative processes. While further work is required to confirm these points, it is possible that the "SASP-resistant" model of IBD under conditions described for optimizing its developed here could be useful in development of more effective disease-modifying drugs.

The light and electron microscopic observations show that the principle pathological changes adjacent to developed ulcers are infiltration of inflammatory leukocytes, the appearance of red blood cells, and associated edema in the interstitial region near the ulcers (Figure 6A and B). The abundance of PMNs, plasmacytes and eosinophils (Figures 6A and B, 7A–D, and Results) is characteristic of the Arthus-like reaction, which is considered presumptive of ulcerative colitis in man (3, 4). Moreover,

some of the similarities of intestinal inflammation in the present study agree with the reported SEM and TEM appearances of this condition in patients (26).

The presence of granular material in the PMNs was particularly striking (Figure 7A–D). This material may be derived from injected carrageenan and/or *Bacteroides* spp. The filamentous-like structures shown in Figure 7B and C is suggestive of this material being carrageenan, yet the section of rod-like granular deposits in Figure 7A and D resemble bacteria. If carrageenan is indeed present in PMNs, this could have consequences for the functions of these cells to produce cell-destructive enzymes and oxyradicals. The carrageenan also would be expected to depress B-cell but not T-cell functions (27). Furthermore, desulfated carrageenans are notably cytotoxic to human macrophages (28), so this might explain the predominance of PMNs and eosinophils compared to cells resembling monocytes or macrophage-like cells in the inflammatory cells. The lack of effects of SASP in this model is a further indication of the limitations of this drug compared with that in other less severe animal models. From these observations, one might speculate that the model is more appropriate for the study of a "chronic SASP-resistant" profoundly immunologic disease.

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