

The Germfree State Prevents Development of Gut and Joint Inflammatory Disease in *HLA-B27* Transgenic Rats

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Summary

A number of inflammatory disease states occur with greatly increased frequency in individuals inheriting the human major histocompatibility complex class I allele *HLA-B27*. In a minority of cases, namely those with B27-associated reactive arthritis, there is good evidence that the disease state is triggered by infection with an enteric or genitourinary bacterial pathogen. For the majority of B27-associated disease, no definite pathogenetic role for bacteria has been established. However, in these latter cases intestinal inflammation can often be demonstrated, and it sometimes occupies a major part of the clinical picture. Rats transgenic for B27 are known to develop a disorder resembling B27-associated human disease, with prominent intestinal, joint, skin, and male genital inflammatory lesions. We report here that B27 transgenic rats raised in a germfree environment do not develop inflammatory intestinal or peripheral joint disease, whereas the skin and genital inflammatory lesions are unaffected by the germfree state. These findings support the concept that gut and joint inflammation are pathogenetically closely related, and they provide direct evidence that the commensal gut flora play an important role in the pathogenesis of B27-associated gut and joint inflammation.

H*HLA-B27* is strikingly associated with the disorders termed spondyloarthropathies (for reviews see references 1 and 2). These disorders are recognized clinically largely on the basis of joint inflammation, and they are therefore classified as rheumatic diseases. However, a large body of evidence suggests an intimate involvement of intestinal inflammation in the pathogenesis of these disorders. This evidence includes triggering of B27-associated reactive arthritis by certain enteric pathogens (3), increased prevalence of ankylosing spondylitis and peripheral arthritis in patients with inflammatory bowel disease (4, 5), increased prevalence of inflammatory bowel disease in family members of patients with ankylosing spondylitis (6), acute intestinal inflammation occurring in the setting of reactive arthritis arising from genitourinary

infection (3, 7), the presence of microscopic bowel inflammation in a high proportion of patients with B27-associated rheumatic disease (8, 9), antigenic and structural cross-reactivity between the *HLA-B27* molecule and components of enteric bacteria (10, 11), diminished internalization of enteric bacteria by cultured fibroblasts expressing *HLA-B27* (12), and triggering of inflammatory arthritis in animals by experimental injection of peptidoglycan from normal bowel flora (13).

This intimate association between the gastrointestinal tract and the spondyloarthropathies is reproduced in transgenic rats expressing *HLA-B27*, in which gut and joint inflammation are prominent aspects of a spontaneous multisystem disease that resembles the human spondyloarthropathies (2, 14, 15). Because of the evidence for a potential role for intestinal flora in the pathogenesis of B27-associated disease, we investigated the effect of the germfree state on the inflammatory disease of B27 transgenic rats. Here we report the results of these studies.

A portion of this work has been reported in abstract form (1993. *Arthritis Rheum.* 36:S46; 1994. *FASEB [Fed. Am. Soc. Exp. Biol.] J.* 8:A968).

Material and Methods

Derivation and Maintenance of Germfree Rats. The transgenic rat lines 33-3 (F344 strain) and 21-4H (LEW strain) expressing genes for HLA-B*2705 and human β 2-microglobulin have been previously described (2, 14). One litter of each line was rederived by cesarean section into a germfree environment at the University of Wisconsin Gnotobiotic Laboratory. These rats and their descendants were maintained in this environment and observed for disease manifestations as described (14). Weekly stool examinations for aerobic and anaerobic bacteria, parasites and fungi, and serology for eight viruses, were uniformly negative. Rats were shipped to the University of Texas Southwestern Medical Center for necropsy or for transfer to a nongermfree environment. Rats were shipped in germfree containers and maintained under sterile conditions until killing or transfer to a nongermfree colony, with the exception of one shipment of nine rats shipped in nongermfree containers.

Nongermfree Rats. Rats not housed under germfree conditions were kept either in a specific pathogen-free barrier facility or in a conventional room.

Northern Blotting. This was carried out as previously described (2, 14). Hepatic α 1-acid glycoprotein (α 1-AGP) transcripts were identified with an 850-bp rat α 1-AGP cDNA fragment (16) (gift of Dr. H. Baumann, Roswell Park Cancer Institute, Buffalo, NY).

Histology, Immunocytochemistry, and Flow Cytometry. These were carried out as previously described (2, 14).

Results

Protection from Gut Inflammation. 37 transgenic rats (19 21-4H and 18 33-3) and 17 nontransgenic littermates (six LEW and 11 F344) were observed under germfree conditions for 23–66 wk. None of these germfree rats showed any evidence of diarrhea, a prominent finding by the age of 20 wk in these transgenic lines when housed under nongermfree conditions (14). Necropsies were performed on 17 germfree 21-4H rats and 13 germfree 33-3 rats, and also on six nontransgenic germfree littermates (three LEW, three F344). All 36 rats showed massive cecal enlargement at necropsy, a well-established sign of the germfree state in rodents that reverses rapidly after association with gastrointestinal flora (17). One 58-wk-old germfree transgenic 21-4H rat showed a peculiar thickening of the large and small intestinal wall, but was free of diarrhea.

Histologically, the colons of these rats showed shallow crypts, another previously described sign of the germfree state in rodents (18). Some of the transgenic colon sections showed a subtle hypercellularity within the lamina propria and slight elongation of crypt height, in comparison with the nontransgenic germfree sections, but no inflammatory lesions were evident and none of the sections appeared outside the range of normal. Fig. 1, A and B show the colon histology typical of the germfree 21-4H and 33-3 rats, respectively. For comparison, the normal colon histology of a nontransgenic germfree LEW littermate control rat is shown in Fig. 1 C, and prominent colitis evident in an age-matched transgenic 21-4H rat born and raised with normal intestinal flora is shown in Fig. 1 D.

Sections of ileum or stomach were examined, respectively, in seven and eight germfree transgenic rats. Mild lymphocytic infiltration of the lamina propria was found in five of

the stomachs. Two ileal specimens showed mild crypt hyperplasia, one of which was from the one rat described above with thickening of the intestinal wall.

Joint Disease. No arthritis or other joint abnormalities were noted clinically at any time in the germfree rats. Ankle joints from four 21-4H and four 33-3 rats, 39–58 wk of age, were examined histologically and found to be completely normal. Although, in our experience, inflammatory lesions have always been histologically evident in joints that showed clinical arthritis, the sensitivity of the absence of clinically evident arthritis to predict normal histology had not been determined. To test this, clinically normal ankle joints from the following rats were examined histologically: 11 line 33-3 rats, 36–57 wk of age, from the conventional or barrier colonies, and three 64-wk-old 21-4H rats transferred at 8 wk of age from the germfree facility to the barrier facility. No arthritis was found in any of these specimens. These results indicate a complete suppression of peripheral arthritis by the germfree state.

Tail vertebral joints were examined histologically from the same eight germfree rats and two of the transferred 64-wk-old 21-4H rats described in the previous paragraph, and from 16 transgenic nongermfree rats, 27–57 wk of age. One of the eight germfree rats and six of the 16 nongermfree rats showed mild periannular fibrosis.

Cutaneous Disease. Despite the lack of gut and joint disease, the germfree transgenic rats developed prominent skin and nail lesions. These included epidermitis and superficial dermatitis in tail skin (Fig. 2 A) and hyperkeratotic nail changes that have been previously described (2). In addition, the germfree rats showed prominent alopecia associated histologically with folliculitis and perifolliculitis with resultant follicular atrophy (Fig. 2 B). This lesion also occurs with increasing age in the majority of nongermfree rats of the 33-3 and 21-4H lines (Yanagisawa, H., J. A. Richardson, J. D. Taurog, and R. E. Hammer, manuscript in preparation). Comparison of histologic lesions in tail skin showed no significant differences between germfree and conventional or barrier-raised transgenic rats of either line (Table 1).

Male Fertility and Genital Inflammation. The germfree transgenic males developed testicular inflammation and infertility similar to that previously described in nongermfree rats (2). This process may be a nonspecific effect of high HLA-B transgene expression, since a similar lesion has now been observed as the sole pathologic manifestation of transgenic rats with high expression of another HLA-B gene, HLA-B7 (Maika, S. D., J. D. Taurog, W. A. Simmons, J. A. Richardson, and R. E. Hammer, manuscript in preparation). One mating between two 33-3 germfree rats produced two offspring homozygous for the transgene locus, as determined by quantitative hybridization of genomic DNA. The phenotype of the two homozygotes did not differ from that of the hemizygous germfree rats.

Expression of HLA-B27 in Germfree Rats. Previously we observed that disease susceptibility in the B27 transgenic rat lines correlated with high B27 expression in lymphoid tissue (14). To examine the possibility that the absence of gut or joint inflammation in the germfree rats might be associated

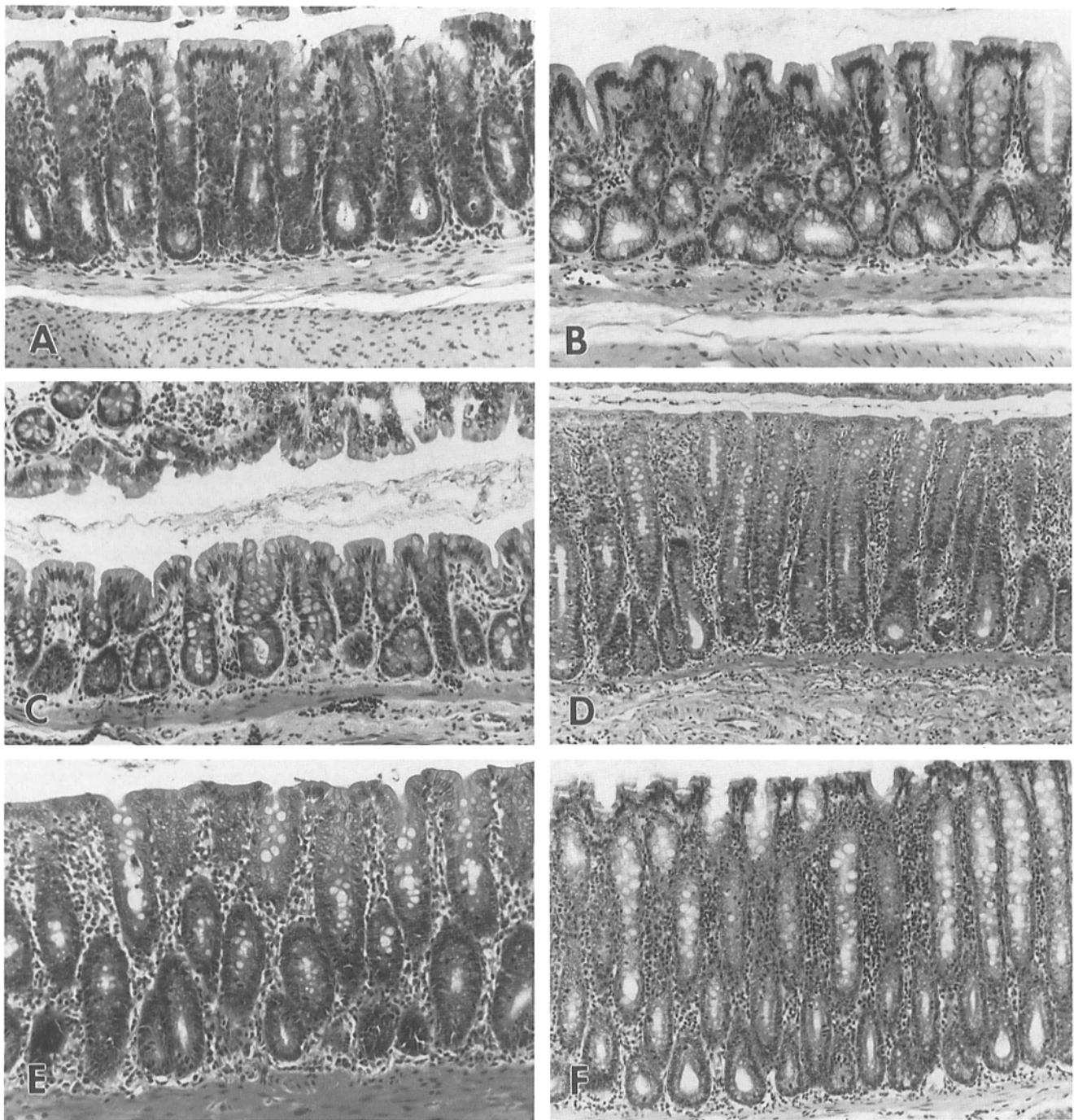


Figure 1. Histology of distal colon in germfree and nongermfree rats. (A) Colon of a 52-wk-old germfree 21-4H male rat. The section shows shallow crypts with no inflammation. (B) Colon of a 39-wk-old germfree 33-3 male rat, with histology similar to that shown in A. (C) Colon of a 48-wk-old germfree nontransgenic male 21-4H littermate, with histology similar to that shown in A. (D) Colon of a 52-wk-old 21-4H female rat born and raised in a barrier facility. The section shows typical chronic colitis, with crypt hyperplasia, loss of mucin-containing cells, and an inflammatory infiltrate in the lamina propria. (E) Colon of a 55-wk-old 21-4H male rat germfree until age 52 wk and then housed in a conventional room for 23 d. The section shows early inflammatory changes. (F) Colon of a 52-wk-old 21-4H male rat germfree until age 8 wk and then housed in a barrier facility for 44 wk. The section shows changes similar to those shown in D (A-C, E) $\times 130$; (D) $\times 73$; (F) $\times 100$.

with diminished expression of B27, flow cytometry was carried out simultaneously on lymphoid cells from germfree and nongermfree transgenic rats. B27 surface expression in the germfree rats consistently equaled or exceeded that of age-

matched nongermfree transgenic rats with severe disease (data not shown). In addition, immunocytochemistry of colon from transgenic germfree rats showed prominent staining for B27 of the resident cells in the lamina propria (data not shown).

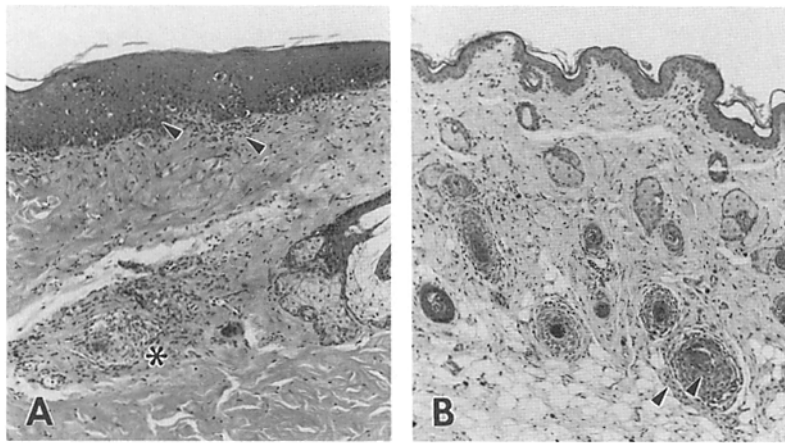


Figure 2. Histopathology of skin from germfree B27 rats. (A) Tail skin of a 23-wk-old germfree 21-4H male rat, showing epidermal hyperplasia and inflammatory infiltration of the epidermis and superficial dermis (arrowheads). Folliculitis and perifolliculitis are evident in the dermis (asterisk) $\times 57$. (B) Trunk skin of a 51-wk-old germfree 33-3 female rat, showing prominent folliculitis and perifolliculitis (arrowheads) $\times 55$.

Suppression of Acute Phase Response. To apply a quantitative assessment to the suppression of inflammation by the germfree state, mRNA transcripts for the acute phase reactant $\alpha 1$ -AGP were measured in liver samples from germfree and nongermfree 33-3 transgenic and F344 control rats. As shown in Fig. 3, the germfree rats showed reduced levels of $\alpha 1$ -AGP message, compared with nongermfree transgenic 33-3 rats.

Reconstitution of Germfree Rats. To examine whether germfree B27 transgenic rats develop gut or joint inflammation once reconstituted with gut flora, two 52-wk-old 21-4H rats were transferred from the germfree facility to the conventional colony. Upon killing 23 d later, both had developed diarrhea, and histologic examination of the colon showed inflammation (Fig. 1 E). None of seven similarly treated non-transgenic LEW or F344 rats showed any clinical or histo-

logic abnormalities (transfers at ages 52–66 wk, observed at 3–14 wk, data not shown). In addition, four 21-4H rats were transferred from the germfree facility to the barrier colony at the age of 8 wk and then killed 16–56 wk later. All four showed histologic evidence of colitis (Fig. 1 F). Early colitis was also seen in a section of colon from one of eight transgenic rats (24–38 wk) that were shipped to Dallas in nongermfree containers 2–3 d before killing, and five of these rats also showed mild crypt hyperplasia of the ileum (data not shown).

Neurologic Disease in 21-4H Rats. Rats of the 21-4H line consistently exhibit a neurologic lesion, beginning in the first month of life, resulting from failure of myelination of populations of axons in the spinal cord and cerebellum (2, and unpublished findings). Similar lesions have been reported in transgenic mice expressing class I MHC genes driven by a

Table 1. Histologic Assessment of Skin

			Histologic appearance		
	<i>n</i>	Age range	Normal	Psoriasiform lesions*	Folliculitis
<i>wk</i>					
Tail skin					
21-4H germfree	12	24-58	3	9	7‡
21-4H nongermfree	4§	24-64	1	2	3
33-3 germfree	9	24-48	1	7	8
33-3 nongermfree	9	36-40	0	5	9
Trunk skin					
21-4H germfree	6	52-58	1	2	5
33-3 germfree	7	40-52	0	2	7

* Dermatitis, acanthosis, keratinocyte necrosis, exocytosis, or parakeratosis.

‡ No follicles were evident in two of the sections.

§ These four rats were transferred from the germfree facility to the nongermfree colony 23–391 d before killing.

|| Age-matched nongermfree specimens were not systematically examined. Lesions were similar to those seen in another study in nongermfree rats (Yanagisawa et al., manuscript in preparation).

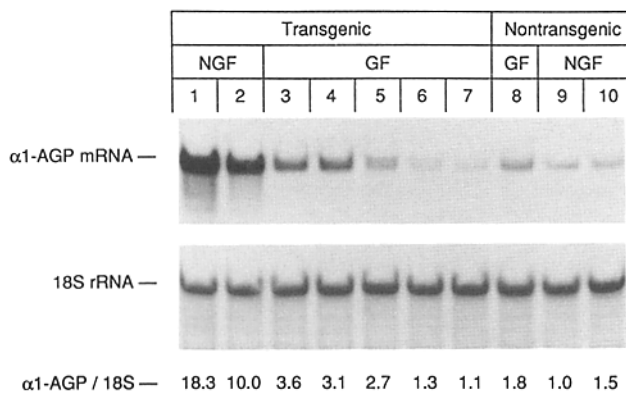


Figure 3. Northern analysis showing $\alpha 1$ -AGP mRNA and 18S RNA expression in livers from line 33-3 rats and nontransgenic littermate controls. The blot was sequentially hybridized with probes for $\alpha 1$ -AGP and 18S RNA and quantitated by phosphorimaging. $\alpha 1$ -AGP/18S RNA ratios, expressed relative to the value for sample 9, are indicated at the bottom. From left to right, lane 1: 51-wk-old 33-3 male, born and raised in a barrier facility; lane 2: 33-wk-old 33-3 female, born and raised in a barrier facility; lanes 3 and 4: 48-wk-old germfree 33-3 females; lane 5: 51-wk-old germfree 33-3 male; lanes 6 and 7: 51-wk-old germfree 33-3 females; lane 8: 48-wk-old germfree nontransgenic female littermate; lane 9: 57-wk-old nontransgenic female littermate, transferred at age 52 wk from germfree to conventional environments; lane 10: 18-wk-old nontransgenic female littermate, raised in barrier facility. (GF) Germfree; (NGF) nongermfree.

myelin promoter (19, 20). Clinical neurologic findings in the germfree 21-4H rats were the same as those seen in nongermfree 21-4H rats.

Discussion

The germfree state protected B27 transgenic rats from gut and peripheral joint inflammation, but had no effect on the progression of skin lesions. In nongermfree transgenic rats, diarrhea usually precedes onset of arthritis (2, 14), and this is also the sequence in enteritis-induced reactive arthritis in humans. Since both gut and joint inflammation were suppressed in the germfree rats, it cannot be discerned with certainty from these experiments whether joint disease arises as a consequence of an intestinal process, or whether both are parallel consequences of one or more antecedent processes related to gut bacteria. Enteric bacterial pathogens are well known to trigger B27-associated rheumatic disease in humans, but a direct role for normal gut flora has not established. The results reported here provide direct evidence that commensal enteric bacteria play a critical role in the pathogenesis

of B27-associated rheumatic disease. The data also support the hypothesis that commensal bacteria play an important role in the pathogenesis of idiopathic inflammatory bowel disease (21). This is also supported by the report that the spontaneous colitis recently described in IL-2-deficient mice was absent in three of three such mice observed in the germ-free state through 5 mo of age (22).

The role of the gut flora in axial arthropathy is somewhat more difficult to assess from these experiments since the only lesions found in any of the rats examined were mild and not necessarily related to inflammation. Lesions were not entirely absent from the germfree rats, but were found with higher prevalence in the nongermfree transgenic rats examined. In humans, ankylosing spondylitis is highly associated with inflammatory bowel disease, but its course is usually independent of the state of the bowel disease, whereas peripheral arthritis tends to parallel the activity of bowel inflammation (23). Similarly, treatment of ankylosing spondylitis patients with sulfasalazine, an agent with documented efficacy in suppressing inflammatory bowel disease, has shown a beneficial effect more consistently on peripheral than on axial arthropathy (24).

These results indicate that the gut flora do not contribute significantly to the pathogenesis of the skin and nail lesions of the B27 rats, in contrast to the effect on gut and joint disease. However, the germfree rats were not housed in an antigen-free environment, and it remains possible that products of nonviable microorganisms might have played a role in the pathogenesis of the cutaneous lesions. This explanation might also account for the subtle differences in gastrointestinal histology observed between transgenic and nontransgenic germfree rats. Alternatively, these findings may be solely manifestations of transgene expression, as we suspect to be the case for the noninflammatory neurologic lesion of the 21-4H line and perhaps also for the male genital inflammation of both lines.

Suppression of gut inflammation was rapidly reversed by removal from the germfree state. No arthritis was seen in the rats with a restored gut flora, but comparatively few rats were examined and the period of observation for some of them was quite short. It thus cannot be concluded from this that arthritis would not appear in this setting if it were systematically sought. Rather, the data suggest that reconstitution experiments with defined bacteria will permit identification of the bacterial components critical to the B27-related disease process.

We thank B. Reese, B. Hastings, R. Hadavand, and S. Montañez for technical assistance.

This work was supported by grants from Glaxo Research Institute and the Crohn's and Colitis Foundation of America, and by National Institutes of Health grants DK-47692 and AR-38319. J. L. Fernández-Sueiro was supported in part by the Fondo de Investigaciones Sanitarias of Spain, grant 94/5074.

Received for publication 12 August 1994.

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