A Major Quantitative Trait Locus on Mouse Chromosome 3 Is Involved in Disease Susceptibility in Different Colitis Models

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Background & Aims: **Mice with a disrupted gene for the G-protein α inhibitory 2 chain (*Gnai2*$^{-/-}$) develop a spontaneous colitis resembling human inflammatory bowel disease. Disease expression differs markedly between inbred strains of mice, indicating genetic control of disease susceptibility. We performed a genome-wide screen to localize the chromosomal regions regulating disease expression.** ***Methods:*** **A total of 284 F2 mice derived from resistant C57BL/6J *Gnai2*$^{-/-}$ mice and susceptible C3H/HeN *Gnai2*$^{-/-}$ mice were analyzed in a genome-wide screen for colitis susceptibility and severity.** ***Results:*** **A highly significant locus on chromosome 3 (*Gpdc1*) contributed to colitis susceptibility and severity (likelihood ratio statistics [LRS] = 32.4; LOD score = 7; $P < 1.0 \times 10^{-5}$). The peak linkage of this locus at 62 cM colocalizes exactly with a previously identified locus controlling colitis susceptibility in interleukin-10–deficient mice. In addition, evidence for linkage with a locus on chromosome 1 (*Gpdc2*; LRS = 19.7; LOD = 4.3) was found, and the 2 loci interacted epistatically (combined LRS = 68.2). A third locus (*Gpdc3*) was found on chromosome 9 and this locus interacted epistatically with a locus on chromosome 7, which by itself did not have an effect on the trait.** ***Conclusions:*** **The identification of a major locus on chromosome 3 that controls susceptibility to spontaneous colitis in 2 different gene-knockout models indicates that this locus harbors a gene(s) that plays a key role in maintaining mucosal homeostasis. Identification of this gene(s) may contribute to further understanding of the mechanisms underlying human inflammatory bowel disease.**

While the exact cause of the 2 major forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis, is still unclear, there is a growing agreement that they occur as the consequence of a genetically determined dysregulated immune response to one or more antigens from the mucosal microflora.[1]

The fact that this abnormality has strong genetic underpinnings has long been recognized, and in recent years a number of IBD susceptibility loci have been established by linkage studies.[2] Moreover, the susceptibility conferred by one of these loci (IBD1) has been shown to be due to mutations in the NOD2 (*CARD15*) gene; when present on both chromosomes, this causes CD in 8%–17% of white patients with CD.[3,4] It is now known that NOD2 is an intracellular sensor of a component of bacterial peptidoglycan (muramyl dipeptide) that regulates nuclear factor κB activation[5]; nevertheless, it is not yet known how this abnormality leads to CD and/or a dysregulated immune response. In addition, the fact that NOD2 mutations occur in only a minority of patients with CD underscores the fact that it accounts for only a small part of the genetic factors underlying CD as a whole and suggests that the mucosal immune deregulation causing this disease can have many causes.

An important step forward in the understanding of IBD has come from the identification of animal models of mucosal inflammation that more or less resemble human IBD.[6] One of the key findings to emerge from the study of these models is that susceptibility to colitis in most, if not all, of the experimental models is influenced by the strain of the mouse in which the model is expressed. These strain differences in disease susceptibility offer the opportunity to identify genes that are involved in determining susceptibility/resistance to mucosal inflammation and, as such, may contribute to the identification of the genes involved in human IBD.

One mouse model that displays such strain differences in susceptibility is the spontaneous colitis model occurring in mice deficient in the G-protein α inhibitory 2 chain (*Gnai2*).[7–10] Such mice most likely develop muco-

Abbreviations used in this paper: GBP1, guanylate binding protein 1; IL, interleukin; LOD, logarithm of the odds; LRS, likelihood ratio statistics; PCR, polymerase chain reaction; QTL, quantitative trait locus; TNBS, trinitrobenzene sulfonic acid.

0016-5085/05/$30.00
doi:10.1053/j.gastro.2004.10.044

sal inflammation because signaling through *Gnai2* normally inhibits interleukin (IL)-12 production and thus, in the absence of this molecule, IL-12 production and the T_h1 response is greatly exaggerated. The strain-specific variation in inflammation observed in this model is exemplified by the fact that the C3H/HeN strain is highly susceptible to disease, whereas the C57BL/6J strain maintained under identical circumstances is highly resistant. As implied above, this introduces the possibility that a genetic analysis of these strains of *Gnai2*$^{-/-}$ mice might lead to the identification of modifying genes critical to the expression of colitis in these mice.

On this basis, we performed such a genetic analysis of *Gnai2*$^{-/-}$ mice using genome-wide microsatellite methodology to identify genetic susceptibility loci in *Gnai2*$^{-/-}$ (C3H/HeNxC57BL/6J) F2 generation intercrosses. This genetic screening procedure allowed identification of a highly significant susceptibility locus on chromosome 3 as well as additional loci on chromosome 1, chromosome 9, and the X chromosome. The importance of the chromosome 3 locus is emphasized by the fact that this locus also is likely to be involved in the strain variation in disease susceptibility found in IL-10 knockout mice.[11]

Materials and Methods

Mice

Specific pathogen–free, 5–6-week-old C3H/HeN mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *Gnai2* knockout mice on the C57BL/6J background were bred from homozygous C57BL/6J breeding pairs as described before[10,12] and were originally generated at Baylor University School of Medicine. The *Gnai2* deficiency was backcrossed from the C57BL/6J background onto the C3H/HeN strain for >6 generations. Because *Gnai2* deficiency causes severe inflammation in C3H/HeN mice, resulting in early death and loss of desire to breed, these mice were maintained in the heterozygous state (*Gnai2*$^{+/-}$).

C57BL/6J × C3H/HeN *Gnai2*$^{-/-}$ F1 hybrids were derived from the reciprocal mating of C57BL/6J *Gnai2*$^{-/-}$ mice with C3H/HeN *Gnai2*$^{+/-}$ mice and subsequent genotypic selection for the *Gnai2*$^{-/-}$ deficiency. These mice were used to generate a first group of 147 F2 mice by brother-sister mating of F1 *Gnai2*$^{-/-}$ mice, and this group of mice was analyzed using a genome-wide screen. Meanwhile, an additional group of 137 F2 mice was generated, for a total of 284 F2 mice (146 female and 138 male).

Mice were maintained in the animal holding facilities of the National Institute of Allergy and Infectious Diseases, and all breeding pairs were set up randomly. Animal use adhered to National Institutes of Health Laboratory Animal Care Guidelines.

Table 1. Definition of Disease Severity in *Gnai2*-Deficient F2 Mice

Score	Histology	% Weight loss	Age at death (*wk*)
0	0	0–4	26+
1	1	5–9	20–25
2	2	10–19	15–19
3	3	20–29	10–14
4	4	30+	0–9

NOTE. Total score of colitis was determined by the sum of the score for each trait (histology, weight loss, age at death) and adding an extra point if an animal was found dead.

Clinical Evaluation and Definition of Disease

Susceptibility to and severity of colitis in F2 mice were determined by weekly monitoring of clinical signs of colitis, diarrhea, and weight loss. Mice were killed when they showed profound weight loss and/or signs of colitis. Mice that showed no signs of disease and no weight loss at the age of 5 months were considered resistant to colitis and were killed as well. Blood was drawn from all mice for serologic studies, and colons were removed for histopathologic analysis.

Histologic Assessment of Tissues

Colonic tissue specimens were obtained from all killed mice and fixed in 10% buffered formalin phosphate (Sigma-Aldrich, Zwijndrecht, The Netherlands). The specimens were embedded in paraffin, cut into sections, and stained with H&E. The degree of inflammation on microscopic horizontal sections of the entire colon was graded semiquantitatively from 0 to 4 as described previously[13] (in short: 0, no evidence of inflammation; 1, low level of lymphocyte infiltration with infiltration seen in 10% high-power fields and no structural changes observed; 2, moderate lymphocyte infiltration with infiltration seen in 10%–25% high-power fields, crypt elongation, bowel wall thickening that does not extend beyond the mucosal layer, and no evidence of ulceration; 3, high level of lymphocyte infiltration with infiltration seen in 25%–50% high-power fields, high vascular density, and thickening of the bowel wall that extends beyond the mucosal layer; 4, marked degree of lymphocyte infiltration with infiltration seen in 50% high-power fields, high vascular density, crypt elongation with distortion, and transmural bowel wall thickening with ulceration).

Definition of Groups

To define a semiquantitative and gradual division of the disease expression in F2 mice, we created a total score of disease severity, combining the percentage of weight loss, the age at the time the mice were killed, and the histologic score by assigning a score from 0 to 4 for each grade of the corresponding trait (Table 1). If mice were found dead (due to rapid disease progression), an extra point was added to the total score. Theoretically, this would result in a maximum score of 13. Animals were considered resistant when they had a total

disease score of 3 or less, implicating they displayed no or only minimal clinical or histologic signs of disease when killed at age 20 weeks or older, whereas mice with a total score of 4 or higher were considered susceptible. We had previously established that mice that had not developed any signs of disease by the age of 20 weeks would not do so if they were followed up for a longer period (up to 1 year; data not shown). Therefore, mice that did not develop any signs of disease were killed at the age of 20 weeks.

In part of the analysis, the phenotypic extremes were analyzed. These included on the one hand most resistant mice with a disease score of 0–2 and on the other hand most susceptible mice with a score of 8 or higher.

Genotyping

Spleens were taken from all mice and frozen at −70°C. DNA was isolated using a commercial kit as described by the manufacturer (Biozym; Gentra Systems, Landgraaf, The Netherlands). Primers for microsatellite markers that differ at least 6 base pairs in size between susceptible C3H/HeN mice and resistant C57BL/6J mice were selected from online available databases (CIDR; http://www.cidr.jhmi.edu/mouse/mouse.html) and The Jackson Laboratory (http://www.informatics.jax.org/) and purchased from Invitrogen Life Technologies (Breda, The Netherlands). A total of 134 microsatellite markers equally dispersed along the mouse autosomes and the X chromosome were selected for genotypic analysis. Using these markers, a genetic map was generated with an average locus distance of 12 cM, with the largest distance (28 cM) on proximal chromosome 2 between markers D2Mit1 and D2Mit242. The markers used in this study will be published online (http://www.immunogenetics.nl).

To evaluate which markers cosegregate with disease phenotype, a first group of 147 mice was selected for a genome-wide screen: 56 resistant F2 mice (30 female and 26 male) with a disease score of 3 or less and 91 F2 susceptible mice (47 female and 44 male) with a disease score of 4 or higher. From this group, 76 mice were selected with the most distinct phenotypes (47 resistant and 29 with severe colitis). Genetic regions that showed suggestive or significant linkage, either from the group with the extreme phenotypes or the total first group, were then further investigated by genotyping the remainder of the eligible mice.

Polymerase chain reaction (PCR) amplification was performed in GeneAmp PCR System 9600 and GeneAmp PCR System 9700 (both PE Applied Biosystems, Niewerkerk aan den IJssel, The Netherlands) in 96-well plates in 10-μL volumes using a protocol provided by the manufacturer (Research Genetics, Invitrogen). Thirty cycles at 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 60 seconds followed by an extension period at 72°C for 7 minutes was generally used, although some primers required a slightly different annealing temperature for optimum amplification. After PCR amplification, PCR products were run on 4% agarose gels (in a mixture of 2% ultrapure agarose [NuSieve GTG Agarose; Cambrex Bio Science Rockland, Rockland, ME]) and 2% low melting agarose (Invitrogen) and visualized by ethidium bromide (Invitrogen) staining.

Statistical Analysis

The MapManager software package was used to construct linkage maps with microsatellite markers that experimentally showed polymorphism between the C3H/HeN and the C57BL/6J strain (http://mapmgr.roswellpark.org/mmQTX.html). Likelihood ratio statistics (LRS) values for colitis severity were calculated with MapManager QTXb18 version 0.27 in populations of females and males, females alone, and males alone. Logarithm of the odds (LOD) scores were determined by LRS/2ln10. Empiric thresholds for suggestive and significant linkage were determined by permutation analysis using the MapManager software package with 1000 permutations. Gene-gene interactions between the different genetic loci were identified using the same statistical program.

For nonparametric linkage analysis of resistant and susceptible mice, χ^2 analysis was performed using GraphPad InStat version 3.05 for Windows 95 (GraphPad Software, San Diego, CA; http://www.graphpad.com). A χ^2 test statistic for each marker locus was derived using 2×3 contingency tables to test for linkage.

Results

Distribution of Colitis Susceptibility in Parental Strains and Their Offspring

Parental strains. We generated homozygous C3H/HeN *Gnai2*-deficient mice by crossing C57BL/6J *Gnai2*-deficient mice with C3H/HeN mice, backcrossing F1 mice carrying the gene defect to C3H/HeN mice for >6 generations, and then intercrossing to obtain homozygous mice. Such homozygous *Gnai2*-deficient ($Gnai2^{-/-}$) C3H/HeN mice, when reared under specific pathogen–free conditions, were highly susceptible to colitis (Figure 1). As previously described, colitis in C3H/HeN $Gnai2^{-/-}$ mice is characterized by severe diarrhea and weight loss, frequently accompanied by rectal prolapse and dilation of the colon.[10] In general, only the distal half of the colon is involved, with a sharp demarcation between the involved and noninvolved areas. Microscopically, massive infiltrations of lymphocytic cells are seen, accompanied by depletion of goblet cells, crypt elongation, high vascular density, and transmural bowel wall thickening with ulceration (Figure 2).

C3H/HeN $Gnai2^{-/-}$ mice display a greatly reduced survival rate and in general breed poorly, probably because most mice develop disease before the reproductive age. Colitis develops in these mice as early as week 6 and, even before full onset of gastrointestinal disease, the animals exhibit decreased body weight as compared with their wild-type littermates (data not shown). Therefore, for breeding purposes, the C3H/HeN mice were main-

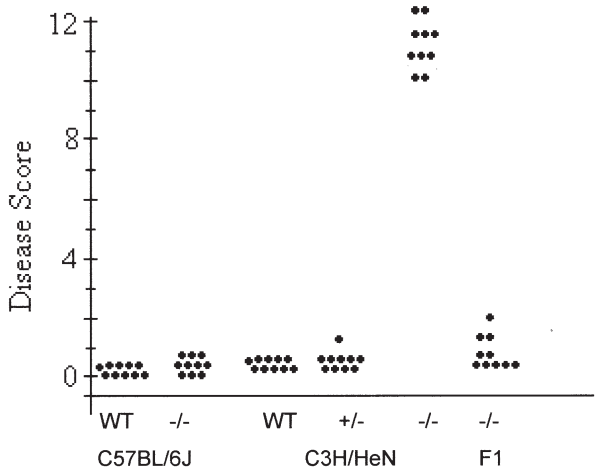

Figure 1. Distribution of colitis scores in different groups of mice. One illustrative experiment out of several, each including 10 mice per group, is shown. Each dot represents a single mouse.

tained in a heterozygous state for the *Gnai2* deficiency ($Gnai2^{+/-}$). Based on genotyping the offspring of $Gnai2^{+/-} \times Gnai2^{+/-}$ mice, the frequency of homozygous *Gnai2*-deficient mice was markedly below the expected 25%, indicating intrauterine death in a significant number of $Gnai2^{-/-}$ mice (results not shown). Interestingly, heterozygous *Gnai2* C3H/HeN-deficient mice did not develop any signs of disease, indicating that colitis only occurs if no functional Gnai2 is present (Figure 1).

Whereas C3H/HeN $Gnai2^{-/-}$ mice are highly susceptible to colitis, C57BL/6J $Gnai2^{-/-}$ mice are highly resistant and disease did not occur in these mice even when the latter were followed up for as long as 6 months (Figure 1); in addition, upon necropsy, colons of these mice were normal, both macroscopically and microscopically (Figure 2*C* and *D*).

F1 generation. The F1 generation was generated by crossing C3H/HeN $Gnai2^{+/-}$ mice with C57BL/6J $Gnai2^{-/-}$ mice. As expected, 50% of the offspring were homozygous for the *Gnai2* deficiency, and these mice were used for further analysis and breeding purposes. The $Gnai2^{-/-}$ F1 offspring were relatively resistant to colitis. Thus, in general, these animals did not develop weight loss, diarrhea, or macroscopic signs of colitis, although occasionally some histologic abnormalities were found (Figure 2*E*).

F2 generation. A total of 284 F2 mice (138 male and 146 female) were generated by interbreeding the $Gnai2^{-/-}$ F1 generation. As can be seen from Figures 2 and 3, the F2 population displayed a broad spectrum of disease, varying from highly resistant with no clinical and histologic signs of illness to highly susceptible with severe colitis. The severity of colitis was determined using a semiquantitative classification as described in detail in Materials and Methods. However, as can also be seen from Figure 3, the trait followed an essentially bimodal distribution. Thus, in part of the analysis, the trait was analyzed as a qualitative trait: mice with a disease score of 3 or less (n = 145; 79 male and 66 female) were considered resistant to colitis because they did not exhibit any clinical signs of disease and, in addition, displayed no or only minimal weight loss and histologic abnormalities (Table 1). In contrast, a total of 139 mice (59

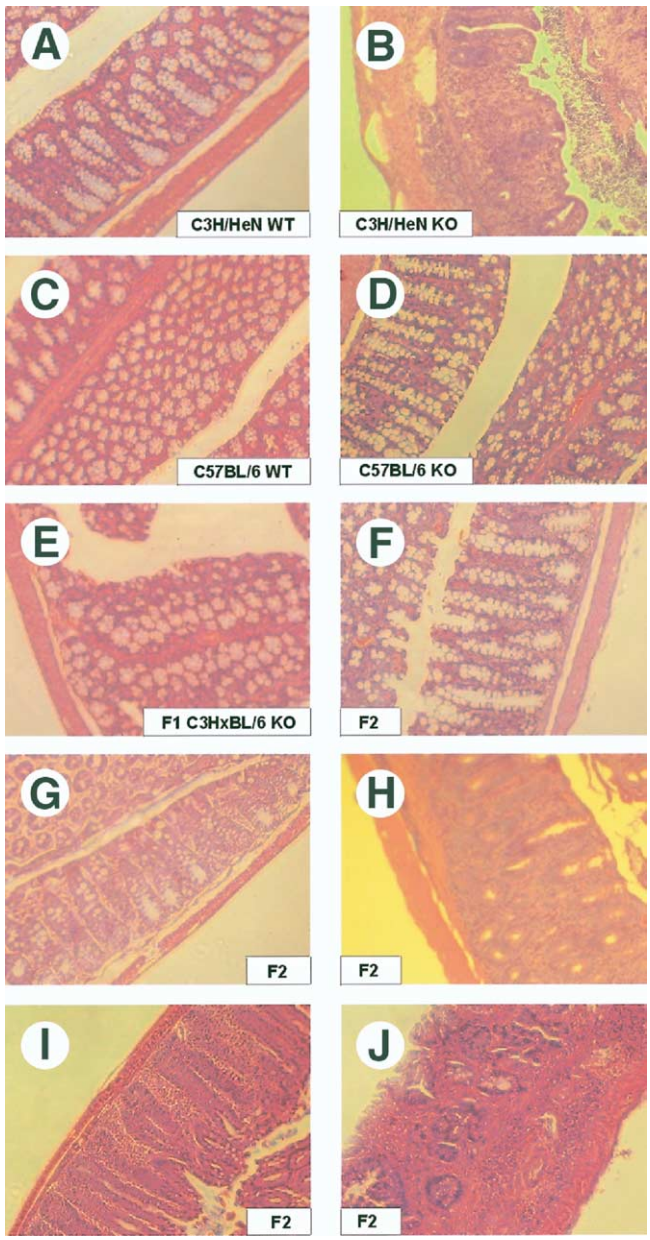

Figure 2. Microscopic views of normal and diseased colons. Representative H&E-stained longitudinal sections of colons of (*A*) wild-type C3H/HeN, (*B*) $Gnai2^{-/-}$ C3H/HeN, (*C*) wild-type C57BL/6J, (*D*) $Gnai2^{-/-}$ C57BL/6J, (*E*) $Gnai2^{-/-}$ C3H/HeN × C57BL/6J F1, and (*F–J*) $Gnai2^{-/-}$ F2 mice, with increasing histologic scores. (Original magnification 100×.)

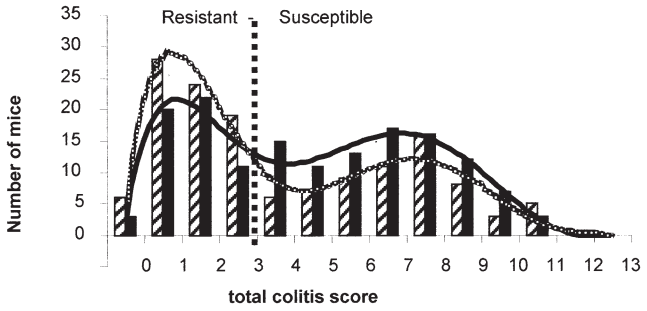

Figure 3. Distribution of colitis in *Gnai2*-deficient F2 mice. The *dotted line* indicates the cutoff for resistant/susceptible mice. The severity of colitis follows a bimodal distribution. Also, female mice are more severely affected than male mice. Hatched bars and lines indicate male mice, solid bars and lines indicate female mice.

male and 80 female) displayed clinical signs of disease accompanied by significant weight loss and histologic disease (ie, a disease score of 4 or higher) and were considered susceptible. Overall, female mice were somewhat more susceptible to colitis than male mice. This was reflected in the mean colitis score, which was higher in female mice than in male mice (4.9 vs 4.2; $P < .05$). Occasionally, histologic signs indicative of colon carcinoma were found in F2 mice. This occurred exclusively in mice with severe inflammation. However, there was no clear correlation between occurrence of carcinoma and the duration of the disease, because, in some instances, neoplastic lesions were found in mice as young as 9 weeks.

Strategies for Analysis and Distribution of Traits

To identify the genetic regions determining the differences in susceptibility between susceptible C3H/HeN and resistant C57BL/6J mice, we genotyped the initial group of 147 F2 mice of our cohort with a panel of 134 microsatellite markers equally dispersed among the genome.

We first analyzed disease in the F2 population as a qualitative (binary) trait (ie, susceptible vs resistant mice). Among the 147 mice, 56 (30 female and 26 male) were relatively resistant, with a disease score of 3 or less, whereas 91 (47 female and 44 male) were susceptible (with a disease score of 4 or higher). Contrasting genotype and allele frequencies of these groups led to the identification of 4 chromosomal regions that exhibited an association suggestive for linkage ($P < 1.6 \times 10^{-3}$ or stronger).[14] These loci reside on chromosomes 1, 3, and 13 and the X chromosome.

Subsequently, the subgroup of mice with the most extreme phenotypes was analyzed. This analysis has the advantage that the phenotypes of these groups are clearly distinct and nonoverlapping and the disadvantage of lower statistical power due to the lower sample number. Contrasting mice with a disease score of 2 or less with those with a disease score of 8 or higher resulted in essentially the same associations. In addition, this analysis allowed identification of an association with a locus on chromosome 9, which was not found in the entire group of mice.

Finally, disease was considered as a quantitative trait in which the genes contribute to the trait in a continuous fashion rather than in a discrete way. This was accomplished by a total disease score, which takes into account histologic score, weight loss, and the age at which the mice were killed (Table 1). Due to the semiquantitative nature of our phenotypes, we established an empiric genome-wide significance level for our data set by performing a permutation analysis. The observed genotypes of the F2 animals were kept constant while the phenotypes were randomly shuffled over the genotype data, thereby efficiently disrupting any genotype-phenotype correlation. Linkage analysis was performed, and the strongest association (expressed as the LRS) in each experiment was recorded. This procedure was repeated 1000 times, and through this method the LRS threshold for 95% significance was calculated to be 16.5. Suggestive linkage was calculated to be above a threshold LRS value of 10.0.

All chromosomes with regions identified with the above strategies that showed at least suggestive linkage were genotyped in the entire cohort of 284 F2 mice to confirm or discard the linkage.

Identification of Major Susceptibility Locus on Chromosome 3

Analysis of the entire F2 population of 284 mice as a qualitative trait confirmed the presence of a region on the distal part of chromosome 3 that is strongly associated with susceptibility to colitis. As can be seen from Table 2, the strongest association was found with microsatellite markers D3Mit316 at 59 cM ($\chi^2 = 29.7$; $P < 1.0 \times 10^{-5}$) and D3Mit348 at 61 cM ($\chi^2 = 29.6$; $P < 1.0 \times 10^{-5}$). Analysis of phenotypic extremes led to a similarly significant association with these markers ($\chi^2 = 32$; $P < 1.0 \times 10^{-5}$, data not shown). When mice were subdivided according to sex, the same significant associations were found in both males and females.

We then analyzed the F2 population as a quantitative trait. As shown in Figure 4, this analysis confirmed the highly significant association with this region. The strongest association was again with the chromosomal region surrounding D3Mit348 at 61 cM. A peak LRS of 32.4, corresponding to a LOD score of 7, was found for this locus. The strength of the association markedly exceeded the threshold for significant linkage (LRS = 16.5) that was determined empirically by permutation analysis. We have

Table 2. Distribution of Genotypes Surrounding the Locus on Chromosome 3 Regulating Susceptibility to Colitis in *Gnai2*-Deficient F2 Mice

Marker	Position (cM)	Genotype	Resistant (*n*)	Susceptible (*n*)	*P*	χ^2
D3Mit307	22	BB	44	32	2.5×10^{-3}	11.9
		BC	60	77		
		CC	21	50		
D3Mit137	35	BB	41	34	1.0×10^{-4}	18.1
		BC	65	65		
		CC	19	60		
D3Mit310	38	BB	40	34	$<1.0 \times 10^{-5}$	21.7
		BC	68	64		
		CC	17	61		
D3Mit189	49	BB	43	30	$<1.0 \times 10^{-5}$	24.3
		BC	65	67		
		CC	17	62		
D3Mit78	55	BB	42	32	$<1.0 \times 10^{-5}$	24.8
		BC	67	65		
		CC	16	62		
D3Mit316	**59**	BB	39	33	**$<1.0 \times 10^{-5}$**	**29.7**
		BC	72	62		
		CC	14	64		
D3Mit348	**61.2**	BB	42	37	**$<1.0 \times 10^{-5}$**	**29.6**
		BC	69	58		
		CC	14	64		
D3Mit147	79.4	BB	39	38	5.0×10^{-4}	15.2
		BC	68	64		
		CC	18	57		
D3Mit19	87.6	BB	39	37	.02	8.0
		BC	64	71		
		CC	22	51		

NOTE. Microsatellite analysis of chromosome 3. Numbers of different genotypes were compared between susceptible and resistant mice in a contingency table using the χ^2 test for independence. Markers at peak association are indicated in bold. BB, homozygous for the C57BL/6J strain; BC, heterozygous; CC, homozygous for the C3H/HeN strain.

provisionally designated this quantitative trait locus (QTL) *Gpdc1*, for G-protein deficiency–induced colitis.

To study the mode of inheritance of this locus, we stratified mice according to their genotype at position D3Mit348 and determined the severity of colitis in these groups. As can be seen in Figure 5, F2 mice that were homozygous for the C75BL/6 allele at this position had a mean (±SEM) colitis score of 4.01 (±.31) and this was not significantly different from mice heterozygous for the C57BL/6J allele at this position, which had a mean score of 4.16 (±.27). This was in sharp contrast with the colitis score in mice homozygous for the C3H/HeN allele at this position (6.40 ± .32; $P < .001$). These findings are consistent with the view that 2 copies of the C3H allele at this position are required to develop colitis and help to explain the resistance in the F1 population of mice.

Genetic Linkage Analysis: Other QTLs

Because the association with the region on chromosome 3 was remarkably strong, this locus might theoretically overshadow associations with other genetic loci that otherwise might have an important effect on the trait. We therefore performed sequential regression analyses in which we stratified for marker D3Mit348. This analysis continued to show evidence suggestive of linkage to colitis susceptibility on chromosomes 1, 9, and X identified in the initial screen (Table 3) but did not reveal additional loci. The locus on chromosome 13 that was previously mentioned to be a locus of suggestive linkage in the first group did not retain any association in the total group of mice and was thus discarded as being a locus of interest.

Chromosome 1. A locus on chromosome 1, denoted by D1Mit215 at 47 cM, showed suggestive linkage to disease susceptibility in the first group of 147 mice. As can be seen in Table 4, analysis of the entire group of 284 mice as a qualitative trait confirmed this finding but did not markedly increase the strength of the association ($P = 1.5 \times 10^{-3}$; $\chi^2 = 13.0$).

QTL analysis essentially confirmed the qualitative trait data, revealing a broad peak of approximately 30 cM surrounding the marker D1Mit308 (Figure 4), with a maximum LRS of 19.7 (17.8 without stratification for D3M348), which corresponds to an LOD score of 4.3. According to the results obtained in the permutation test, this LRS value exceeds the threshold for significance in a genome-wide screen, and we have therefore provisionally designated this locus *Gpdc2*.

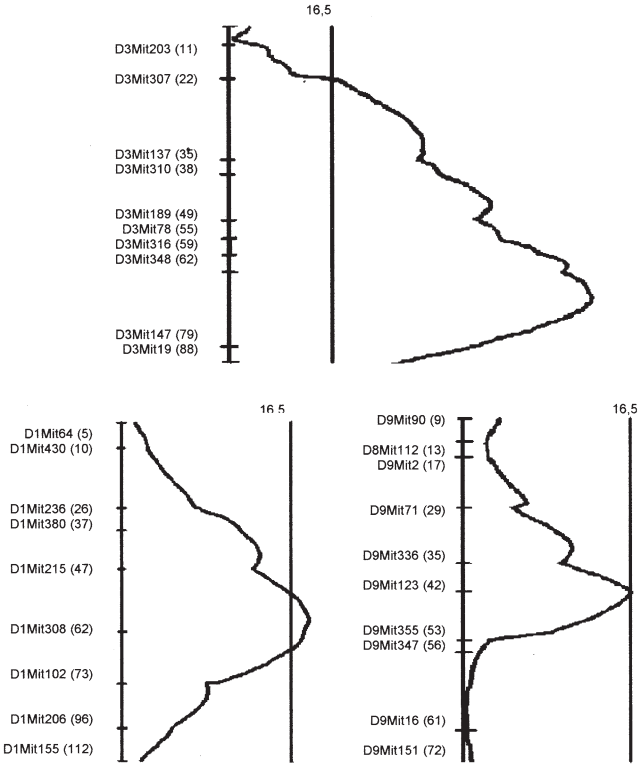

Figure 4. Quantitative trait analysis of chromosomes 1, 3, and 9. On chromosome 1, after stratification by *Gpdc1*, a broad peak is found, spanning a region of approximately 30 cM, from 39 cM to 70 cM (*Gpdc2*). The peak has an LRS of 19.7. On chromosome 3, a very broad region shows linkage to colitis susceptibility, with the 95% confidence interval of 22 cM around the peak marker D3Mit348, which had an LRS of 32.4 (*Gpdc1*). After stratification by *Gpdc1*, on chromosome 9 a peak at 42 cM with marker D9Mit123 just reaches the significance cutoff LRS of 16.5 as indicated by the vertical line (*Gpdc3*). All susceptibility loci are C3H/HeN derived.

Similar to *Gpdc1*, only mice that were homozygous for the C3H/HeN allele showed a significant increase in colitis score, as can be seen in Figure 5. Mice that were homozygous for the C57BL/6J allele and mice that were heterozygous at this locus had a mean (±SEM) colitis score of 4.34 (±.36) and 4.31 (±.27), respectively, whereas mice that were homozygous for the C3H/HeN allele had a mean score of 5.77 (±.33; $P < .01$), indicating that this locus also acts in a recessive manner.

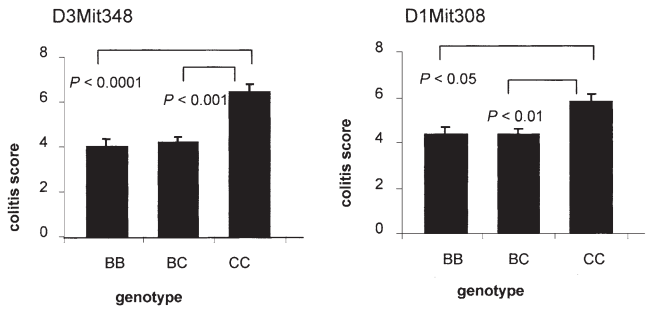

Figure 5. Distribution of colitis score for different genotypes at D3Mit348 and D1Mit308. A significant difference between the C57BL/6J homozygous and C3H/HeN homozygous groups of mice for markers D3Mit348 and D1Mit308 was found. BB, homozygous for the C57BL/6J strain; BC, heterozygous; CC, homozygous for the C3H/HeN strain.

Table 3. Single-Point QTL Analysis for Susceptibility to Colitis in *Gnai2*-Deficient F2 Mice

Locus	LRS
Primary linkage analysis	
D1Mit380	*11.5*
D1Mit215	*12.8*
D1Mit308	**17.8**
D1Mit102	9.7
D3Mit137	**18.4**
D3Mit310	**20.4**
D3Mit189	**24.1**
D3Mit78	**26.0**
D3Mit316	**30.0**
D3Mit348	**32.4**
D3Mit147	10.1
D9Mit336	7.4
D9Mit123	*14.2*
D9Mit355	5.3
DXMit223	*12.9*
Stratified by D3Mit348	
D1Mit380	*11.2*
D1Mit215	*10.6*
D1Mit308	**19.7**
D1Mit102	9.4
D3Mit137	—
D3Mit310	—
D3Mit189	—
D3Mit78	7.9
D3Mit316	8.2
D3Mit348	Not informative
D3Mit147	7.8
D9Mit336	9.6
D9Mit123	**16.5**
D9Mit355	6.8
DXMit223	*14.4*

NOTE. LRS were calculated using marker regression analysis without control for the major QTL on chromosome 3 (primary linkage analysis) or after control for this QTL (stratified by D3Mit348). Suggestive linkage: LRS > 10.0 (in italics); significant linkage: LRS > 16.5 (in bold). All susceptibility loci are C3H/HeN derived.

Chromosome 9. An association suggestive of linkage with chromosome 9 was found in the phenotypic extremes in the first group of mice. Although analysis of the entire group of mice as a qualitative trait did not confirm this finding, a significant deviation from the normal genotype distribution was found in the phenotypic extremes of the entire group ($P = 8.0 \times 10^{-4}$). QTL analysis of the entire group of mice revealed suggestive linkage with marker D9Mit123 at 42 cM, with an LRS of 14.2. After controlling for D3Mit348, the LRS increased to 16.5 and thus reached borderline genome-wide significance (Figure 4 and Table 3). We have provisionally named this C3H/HeN-derived locus *Gpdc3*.

Table 4. Distribution of Genotypes Surrounding the Locus on Chromosome 1 Regulating Susceptibility to Colitis in *Gnai2*-Deficient F2 Mice

Marker	Position (cM)	Genotype	Resistant (*n*)	Susceptible (*n*)	*P*	χ^2
D1Mit64	5	BB	15	25	.86	.3
		BC	25	52		
		CC	10	20		
D1Mit430	10	BB	15	22	.58	1.1
		BC	24	54		
		CC	11	21		
D1Mit236	25.7	BB	12	25	.24	2.8
		BC	29	43		
		CC	9	28		
D1Mit380	36.9	BB	32	35	.074	5.2
		BC	68	73		
		CC	25	51		
D1Mit215	47	BB	34	37	**1.5×10^{-3}**	**13.0**
		BC	68	62		
		CC	23	60		
D1Mit308	62.1	BB	37	33	.018	8.0
		BC	63	71		
		CC	25	55		
D1Mit102	73	BB	37	29	.074	5.2
		BC	63	90		
		CC	25	40		
D1Mit206	95.8	BB	17	24	.48	1.5
		BC	20	46		
		CC	13	27		
D1Mit155	112	BB	14	26	.79	.5
		BC	22	48		
		CC	14	23		

NOTE. Microsatellite analysis of chromosome 1. Numbers of different genotypes were compared between the susceptible and resistant mice in a contingency table using the χ^2 test for independence. Markers at peak association are indicated in bold. BB, homozygous for the C57BL/6J strain; BC, heterozygous; CC, homozygous for the C3H/HeN strain.

X chromosome. Because male mice are hemizygous for the X chromosome, we performed nonparametric analysis for the allele rather than the genotype frequencies in the qualitative trait analysis (Table 5). This resulted in a *P* value suggestive of linkage ($P = 3.0 \times 10^{-3}$) in the total group of mice for marker DXMit223 located distally (at 73.3 cM) on the X chromosome. Separate analysis of male and female mice revealed a stronger association of this locus in female mice than in male mice, although the association in female mice was weaker than in the group as a whole, probably because of the lower statistical power arising from the smaller size of the female subgroup. After stratification for D3Mit348, QTL analysis again revealed a statistical association suggestive of linkage for the total group of mice, with an LRS of 14.1 (Table 3). Again, this susceptibility locus was derived from the C3H/HeN strain.

Epistatic and Additive Interactions With the Identified Locus on Chromosome 3

Separately, the QTLs on chromosomes 1 and 3 and to a lesser extent the loci on chromosomes 9 and X contributed significant main effects to both the qualitative and quantitative disease trait. The MapManager software package was then used to assess epistatic and

Table 5. Allele Frequencies on the X Chromosome in *Gnai2*-Deficient F2 Mice

Marker	Position (cM)	Allele	Resistant (*n*)	Susceptible (*n*)	*P*	χ^2
DXMit68	17.2	B	84 (43)	99 (77)	.06 (.27)	3.5 (1.1)
		C	90 (59)	154 (121)		
DXMit64	45	B	82 (43)	100 (66)	.1 (.2)	2.4 (1.4)
		C	92 (59)	153 (122)		
DXMit223	73.3	B	91 (49)	88 (55)	3.0×10^{-4}	13.0 (10.1)
		C	83 (53)	165 (133)	(1.4×10^{-3})	

NOTE. Microsatellite analysis of the X chromosome. Allele frequencies were compared between susceptible and resistant mice in a contingency table using the χ^2 test for independence. Markers at peak association are indicated in bold. Numbers in parentheses represent female mice. B, C57BL/6J alleles; C, C3H/HeN alleles.

Table 6. Summmary of Epistatic Effects

Locus A	Position (cM)	Individual effect (LRS)	Locus B	Position (cM)	Individual effect (LRS)	Combined effect (LRS)
Chromosome 3 *Gpdc1*	62.1	32.4	Chromosome 1 *Gpdc2*	61.2	17.8	68.2
Chromosome 7 (D7Mit350)	41	4.4	Chromosome 9 *Gpdc3*	42	14.2	37.8

NOTE. Interactions between individual markers were calculated using the MapManager QTX software package. The interacting region of *Gpdc1* conferred D3Mit316 and D3Mit348, and *Gpdc2* conferred D1Mit308 and D1Mit215. Only the highly significant results are tabulated ($P < 1.0 \times 10^{-5}$). All susceptibility regions were C3H/HeN derived.

additive interactions between *Gpdc1* and other chromosomal regions. Only mice that are genotyped for their complete genome are informative for identification of interactions between chromosomal regions; therefore, in this analysis, we used the data from the initially screened 147 mice.

When marker alleles for *Gpdc1* were paired with those of *Gpdc2*, a strong interaction was observed between the 2 loci. Thus, the combined LRS for both loci was higher than the sum of the LRS of both QTLs individually (Table 6; combined LRS = 68.2). In addition, a C3H/HeN-derived locus on chromosome 7, which did not have an effect on the trait individually (LRS = 4.4), showed significant epistatic effects when paired with *Gpdc3* (Table 6).

Distribution of C3H/HeN and C57BL/6J Alleles in the F2 Study Population

The *Gnai2* deficiency of C57BL/6J mice was transferred to C3H/HeN mice by serial backcrossing for 6 generations (see Materials and Methods); this plus the fact that parental C3H/HeN $Gnai2^{-/-}$ mice exhibited a reduced survival rate introduced the possibility that the C3H/HeN *Gnai2* knockout mice carry some excess C57BL/6J DNA that skewed the allele distribution in the F2 generation and thus decreased the number of fully informative mice in this generation.

To determine if this was in fact the case, we first tested the genome-wide allele distribution by comparing the found allele distribution with the expected distribution in a 2×2 contingency table. The genome-wide distribution of C57BL/6J to C3H/HeN alleles did not differ from the expected 1:1 distribution. We then examined the allele distribution in individual chromosomes. For chromosomes 2 and 6, C57BL/6J alleles were slightly more expressed, whereas for chromosomes 4, 8, 14, and X, C3H/HeN alleles were slightly more expressed ($P \leq .05$); however, the overexpression of C57BL/6J alleles was strongly significant only on chromosome 6 ($P = 6.0 \times 10^{-4}$) (data not shown). Thus, the possibility exists that chromosome 6 could harbor a susceptibility region that was inaccessible to discovery in this study.

Discussion

During the past decade, numerous animal models have been identified that have provided key insights into the mechanisms underlying both experimental mucosal inflammation and human IBD.[6] One of the most important of these insights is that the development of colitis in mice with a severe primary immunologic defect (such as IL-10 deficiency[11]) or in mice with colitis induced by a haptenating agent (such as trinitrobenzene sulfonic acid [TNBS]-induced colitis[15]) manifest varying levels of colitis depending on their genetic background. This indicates that even a severe (genetically determined) primary defect may depend on the presence of genetically determined secondary factors to produce disease. This is borne out in the present study of colitis development in $Gnai2^{-/-}$ mice wherein we show that disease expression is dependent on a secondary factor(s) encoded by one or more genes within susceptibility loci on chromosome 3 and other chromosomes.

A second important insight derived from the animal models is that despite the fact that the causes of inflammation differ widely among the various models, they are almost always due to either an exaggerated T_h1 response characterized by high expression of IL-12 and interferon γ or, less frequently, a T_h2 response mediated by IL-4/IL-13; in addition, both types of responses are dependent on cell stimulation by antigens in the mucosal microflora.[6] In the $Gnai2^{-/-}$ mice studied here, the inflammation falls into the T_h1 category in that the colitis is characterized by a marked increase in T_h1-type cytokine production.[16] In this case, the T_h1 orientation is best explained by the studies of He et al, who have shown that treatment of normal mice with pertussis toxin, a substance that inhibits Gi-protein signaling, enhanced the capacity of splenocytes to produce IL-12 in response to both microbial and nonmicrobial stimuli.[12] Thus, the genetically determined absence of Gi signaling in these mice could result in a resetting of the T_h1/T_h2 "immunostat" in a way that leads to an excess T_h1 in response to microbial antigens in the gut. This formulation is consonant with our previous study that provided strong circumstantial evidence that in the TNBS colitis model, a

strain-specific deregulation in IL-12 secretion is present that results in overproduction of IL-12 in response to TLR4 stimulation.[15] In addition, it is consonant with the T_h1 colitides occurring in both IL-10–deficient mice and Stat3-deficient mice (the latter characterized by an IL-10 signaling abnormality), although in these cases T_h1 overproduction is likely due to deficient down-regulation of the mucosal T_h1 responses elicited by mucosal microflora.[17,18] If we now combine the concept of a reset immunostat with the fact noted above that in most cases models of colitis exhibit marked strain specificity and thus evidence for the existence of secondary genetic factors, we can say that the latter is in some manner directly or indirectly influencing the increase in T_h1 response to induce disease. Alternatively, it is creating conditions under which an augmented T_h1 response leads to colitis.

As a first step to identify the genetic factors operating in *Gnai2*$^{-/-}$ mice, we performed a genome-wide linkage analysis for colitis susceptibility. We analyzed F2 intercross mice derived from segregating crosses between relatively resistant C57BL/6J and susceptible C3H/HeN mice. This allowed the identification of several chromosomal loci that are involved in colitis susceptibility and/or severity. The locus that is most strongly associated with susceptibility to colitis, provisionally named *Gpdc1*, resides on the distal end of mouse chromosome 3 and represents the major determinant of susceptibility to colitis in this intercross population. Remarkably, this locus was previously found to control colitis susceptibility in a different model of spontaneous colitis: the IL-10–deficient mouse model.[11] By using virtually the same mouse stocks (the C57BL/6J strain and in this case the C3H/HeJBir strain [which carries the lps mutation in the Toll-like receptor 4 and therefore lacks the capacity to respond to lipopolysaccharide[19]] rather than the C3H/HeN strain), Farmer et al identified a major colitogenic locus (designated *Cdcs1*) at precisely the same position on chromosome 3 as *Gpdc1*. This finding strongly suggests that this region on chromosome 3 harbors a gene that has a strong modulating effect on the exaggerated T_h1 responses seen in both models, which is independent of the primary genetic abnormality that gave rise to these responses. Thus, identification of this gene may provide new fundamental insights in the regulation of pathologic T_h1 mucosal immune responses.

Whether the modifying gene on chromosome 3 described here and previously in IL-10 knockout mice is acting in relation to 2 very different primary abnormalities, operative in each type of mice, or a single abnormality operative in both types of mice is an open question. The first possibility is supported by the findings of He et al, already alluded to above, which locates the defect in the capacity of dendritic cells in *Gnai2*$^{-/-}$ mice to produce IL-12 and not in the regulation of a normal capacity to produce IL-12, as in IL-10 knockout mice. Thus, these data suggest that *Gnai2*$^{-/-}$ mice and IL-10 knockout mice have 2 basically different immunoregulatory abnormalities. The second possibility is favored by data recently reported by Dalwadi et al, who showed that *Gnai2*$^{-/-}$ mice are characterized by a reduction in marginal zone, transitional type 2, and B-1a splenic B cells (which have the capacity to produce IL-10) as well as decreased in vitro (lipopolysaccharide-induced) B-cell IL-10 responses.[20] Thus, these data suggest that *Gnai2*$^{-/-}$ mice also have an IL-10 defect and that the modifying gene can be affecting the IL-10 production defect in both mouse strains. In view of these somewhat disparate findings, it is too soon to decide whether the modifying gene on chromosome 3 is modifying a single abnormality or 2 separate abnormalities.

As previously discussed by Farmer et al, there are several genes present in the identified locus on chromosome 3 that would make attractive candidate secondary disease genes.[11] One of these is the gene encoding the nuclear factor κB p105 transcription factor (*Nfkb1*). Nuclear factor κB designates a group of critical transcription factors controlling various promoters of proinflammatory cytokines, cell-surface receptors, transcription factors, and adhesion molecules.[21] Thus, any mutation affecting the *Nfkb1* gene would clearly have major implications for a great variety of immunologic and/or inflammatory processes. The fact that IL-12 is a cytokine that is under transcriptional regulation of nuclear factor κB makes this gene even more of interest, because in the *Gnai2*$^{-/-}$ model, the IL-12 expression is clearly altered/elevated.[12] Indirect evidence that this gene might be important in IBD comes from a recent study by Karban et al, who found an elevated frequency for the −94delATTG allele in the promoter region of *Nfkb1* in patients with ulcerative colitis,[22] a finding since confirmed by studies in our own IBD patient group (data not shown). Nevertheless, we did not find a polymorphism in the coding region of murine *Nfkb1*, so that if this gene is a disease gene, the abnormality would have to consist of a subtle change in the way it is activated and thus in the way it functions under different circumstances. This multifaceted question is currently under investigation.

The gene encoding epidermal growth factor (the *Egf* gene) is a second candidate gene of interest present in the chromosome 3 locus. Because epidermal growth factor induces epithelial cell proliferation, polymorphisms or mutations in the *Egf* gene could conceivably affect the repair mechanisms of the mucosa and thus the susceptibility to mucosal injury due to inflammatory processes. Indeed, treatment of patients with ulcerative colitis with epidermal growth factor has proven useful in some cases.[23] Yet a third

candidate in the chromosome 3 locus is the gene encoding guanylate binding protein 1 (GBP1), a member of a family of proteins induced by interferon gamma during macrophage induction that shares the ability to bind to guanine nucleotides.[24] GBP1 is a guanosine triphosphatase that converts guanosine triphosphate to guanosine 3′,5′-monophosphate. GBP expression is abundant in interferon-exposed cells, but little is known about its function.[25] Interestingly, type I and II interferons are capable of inducing the synthesis of GBP1 in some strains of mice, including the colitis-susceptible C3H/HeN strain, whereas cells of other strains, including C57BL/6J, fail to synthesize GBP1 in response to both types of interferons and therefore could comprise an important candidate gene.[26]

While the association of disease in *Gnai2*$^{-/-}$ mice with chromosome 3 was quite strong, this locus explained only part of the phenotypic variance. Thus, it is not surprising that we identified other disease susceptibility loci as well. One such locus (designated *Gpdc2*) was found on chromosome 1 at 47 cM and provided strong evidence of interaction with *Gpdc1*. That is to say that the strength of the association of the 2 loci taken together was markedly higher than the sum of the association of the individual loci. This indicates that the 2 loci may act in a combined fashion in determining disease susceptibility. Interestingly, evidence for the presence of a disease gene on chromosome 1 associated with the gene on chromosome 3 (*Cdcs 1*) was also found in IL-10–deficient mice.[11] It should be noted, however, that in this case, peak linkage was more proximal and it thus cannot be excluded that the 2 identified chromosome 1 regions represent different loci.

A third susceptibility locus, *Gpdc3*, was identified on chromosome 9 with peak linkage at 42 cM. At this junction, it is important to note that *Gnai2* also resides on chromosome 9. However, this gene is located at 59 cM, clearly separated from the region of peak linkage, strongly diminishing the possibility that this locus is caused by artificial interference in the knockout backcross protocol. Furthermore, D9Mit355, located at 53 cM, right in between *Gpdc3* and *Gnai2*, is polymorphic for C57BL/6J, C3H/HeN, and 129/SvJ, the strain on which the Gnai2 knockout model was originally created.[10] Using this marker, no residual 129/SvJ DNA was found at this position, adding further evidence that *Gpdc3* is a true QTL (data not shown) and that it is unlikely that this QTL is caused by artificial interference with the *Gnai2* congenic segment. Although *Gpdc3* had only borderline significance for linkage with disease, it is of interest because its location corresponds to a locus we previously identified in the TNBS colitis mouse model, *Tnbs1*.[15] In addition, a similar locus (designated *Ibdq1*) has recently been found by Kozaiwa et al in SAMP1/YitFc mice who spontaneously develop an ileitis similar to that in human CD.[27] Finally, in the DSS model of induced colitis, evidence for the involvement of chromosome 9 has also been obtained.[28] As has been outlined in the study by Kozaiwa et al, precise localization of genes controlling complex traits using F2 and first backcross animals is generally not possible because of the limited number of informative recombinations produced in cohorts of manageable size and the substantial variability of phenotypic expression in mice with random segregations of other disease-associated alleles. However, it is at least striking that several models have disclosed linkage in overlapping regions on mid-chromosome 9, which might indicate that this region does in fact harbor a susceptibility gene that mediates overall susceptibility to intestinal inflammation. Studies in congenic mouse stocks will be able to shed light on the question as to whether the same gene(s) is (are) involved in the different colitis models.

The fact that genome-wide analysis of experimental models has the power to eventually identify disease-causing gene(s) was recently demonstrated by McIntire et al.[29] In their study, they used congenic stocks for a genetic region on mouse chromosome 11 initially identified in a genome-wide screen and were able to identify a gene involving asthma susceptibility using positional cloning.

In conclusion, we have identified a major susceptibility locus on mouse chromosome 3 that exerts its effect in conjunction with a locus on chromosome 1. The identification of similar if not identical loci in different models of spontaneous mucosal inflammation indicates that these regions harbor genes that play an important role in maintaining overall mucosal homeostasis. Identification of these genes, using the approach described above involving congenic mice, will provide detailed understanding of the factors that facilitate the development of colitis in individuals prone to mount increased T_h1 responses. This will thus be relevant to the further understanding of human IBD.

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Received October 16, 2003. Accepted October 7, 2004.

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Supported by the Crohn's and Colitis Foundation of America. G.B. is a Fellow of the Royal Dutch Academy of Arts and Sciences.