A major quantitative trait locus on chromosome 3 controls colitis severity in IL-10-deficient mice


*The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609; and †Department of Medicine, Division of Gastroenterology, University of Alabama, 703 19th Street South, Birmingham, AL 35294-0007

Edited by Charles A. Dinarello, University of Colorado Health Sciences Center, Denver, CO, and approved September 19, 2001 (received for review May 23, 2001)

Colitic lesions are much more severe in C3H/HeBir (C3H) than C57BL/6J (B6) mice after 10 backcrosses of a disrupted interleukin-10 (Il10) gene. This study identified cytokine deficiency-induced colitis susceptibility (Cdcs) modifiers by using quantitative trait locus (QTL) analysis. A segregating F2 population (n = 408) of IL-10-deficient mice was genotyped and necropsied at 6 weeks of age. A major C3H-derived colitogenic QTL (Cdcs1) on chromosome (Chr.) 3 contributed to lesions in both cecum [logarithm of odds ratio (LOD) = 14.6] and colon (LOD = 26.5) as well as colitis-related phenotypes such as spleen/body weight ratio, mesenteric lymph node/body weight ratio, and secretory IgA levels. Evidence for other C3H QTL on Chr. 1 (Cdcs2) and Chr. 2 (Cdcs3) was obtained. Cdcs1 interacted epistatically or contributed additively with loci on other chromosomes. The resistant B6 background also contributed colitogenic QTL: Cdcs4 (Chr. 8), Cdcs5 (Chr. 17), MMQ, and Cdcs6 (Chr. 18). Epistatic interactions between B6 QTL on Chr. 8 and 18 contributing to cecum hyperplasia were particularly striking. In conclusion, a colitogenic susceptibility QTL on Chr. 3 has been shown to exacerbate colitis in combination with modifiers contributed from both parental genomes. The complex nature of interactions among loci in this mouse model system, coupled with separate deleterious contributions from both parental strains, illustrates why detection of human inflammatory bowel disease linkages has proven to be so difficult. A human ortholog of Cdcs2 (Cdcs5) and Chr. 2 (Cdcs4) was obtained.

Materials and Methods

Mice. Development of the C3H and B6 stocks homozygous for a targeted mutation of the Il10 gene (formal designation Il10tm1Cgn) and their reciprocal F1 hybrids have been described previously (15). In the present study, F1 offspring were intercrossed to generate a total of 203 (102 females and 101 males) (C3H.Il10−/− × B6.Il10−/−)F2 and 208 (103 females and 105 males) (B6.Il10−/− × C3H.Il10−/−)F2 mice. Mice were maintained in a humidity-, temperature-, and light cycle (12:12)-controlled vivarium under specific pathogen-free conditions. Opportunistic microbes present in the room were Helicobacter sp. and Pasteurella pneumotropica. Mice were caged in double-pen polycarbonate cages (330-cm2 floor area) and separated by sex at a

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Abbreviations: IBD, inflammatory bowel disease; Chr., chromosome; B6, C57BL/6J; C3H, C3H/HeBir; QTL, quantitative trait locus/loci; Cdcs, cytokine deficiency-induced colitis susceptibility; cM, centimorgan; ECM, Escherichia coli membrane; LOD, logarithm of odds ratio; LPS, lipopolysaccharide.

‡To whom reprint requests should be addressed. E-mail: ehl@jax.org.

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maximum capacity of five mice per pen. Mice were allowed free access to autoclaved food (NIH diet 31, 6% fat) and autoclaved water (pH 2.8–3.2). At 6 weeks of age, females and males were necropsied for tissue collection and analyzed for various phenotypes positively correlated with the progression of colitis. This time point was chosen because the parental strains already showed highly significant differences in histopathologic lesions at this time point (15).

The Il10 gene is located on Chr. 1 at 69.9 centimorgans (cM). Because the gene targeting was done in 129/Ola embryonic stem cells (13) and because the 129 inbred strain background is known to harbor colitis susceptibility modifier genes (14), it was essential to define the length of the congenic segment carrying strain 129 alleles in linkage disequilibrium to the disrupted Il10 gene in both C3H and B6 congenic stocks. The strain 129-derived segment in the C3H congenic stock spanned at least a 4.3-cM region from D1Mit10 (56.6 cM) to D1Mit166 (100 cM). In the B6 congenic stock, the strain 129-derived region spanned at least an 18.6-cM region from D1Mit415 (51.4 cM) to D1Mit445 (70 cM).

Histology and Colitis Assessment. Once the mice reached 6 weeks of age, they were necropsied after CO2 asphyxiation and prepared for histology as described previously (17). The cecum and colon from each mouse were separated, then distended by intraluminal injection using Telly’s acid alcohol formalin, and immersed in fixative overnight. Two hematoxylin and eosin-stained sections of the cecum and one of the colon were coded and reviewed by a pathologist (J.P.S.) who had no prior knowledge of the code. Histology of the cecum and colon was scored separately by using criteria described previously (15); these criteria included severity, hyperplasia, ulceration, and the percentage of area involved. Further, the colon was examined and graded as three individual areas: proximal, middle, and distal. Total scores were determined for the cecum and colon by adding the values of lesions associated with the severity, hyperplasia, ulceration, and percentage of tissue involved. Cecum total score ranged from 0 to 12. Colon total score ranged from 0 to 36, because individual scores from the proximal (0–12), middle (0–12), and distal (0–12) regions of the colon were combined. The lesion severity, hyperplasia, ulceration, and percentage of area involved were graded as follows: 0 = normal, 1 = mild, 2 = moderate, and 3 = severe.

Genotyping. DNA was isolated from 5-mm tail clips and frozen kidneys by using phenol/chloroform extraction. DNAs for all F2 segregants were genotyped by PCR using microsatellite markers purchased from Research Genetics (Huntsville, AL). Cyclers (MJ Research, Cambridge, MA) were used, and products were separated on 4% Metaphor/LE (3:1) agarose gels (BMA Biochemicals). The Jackson Laboratory’s allele typing service also provided multiplex genotyping data by using ABI 370 instrumentation. Ninety-four informative microsatellite markers distinguishing C3H and B6 were used, providing coverage at least every 20 cM on each autosome and Chr. X (18). The list of markers is available on request. The linkage maps and marker positions reported are based on The Jackson Laboratory’s online Mouse Genome Informatics resource (www.informatics.jax.org).

Flow Cytometric Analysis of Blood Leukocytes. One day before necropsy, ~150 μl of blood was collected from the retro-orbital venous plexus for analysis of leukocyte populations by flow cytometry. Whole blood was placed in 1 ml of Hanks’ buffered salt solution containing 5 mM EDTA and then put on ice. Red blood cells were lysed in 3 ml of Gey’s solution, centrifuged at 1,500 rpm for 5 min (Sorvall model RT6000), and repeated a second time. The following monoclonal antibodies were used in combination for leukocyte staining (PharMingen or The Jackson Laboratory flow cytometry service): e; 3-labeled anti-CD4 mAb ( GK1.5-Jackson), FITC-labeled anti-CD8a mAb (53–672), and phycoerythrin-labeled anti-CD3 mAb (145–2C11-Jackson); FITC-labeled anti-B220 mAb (RA3–6B2), phycoerythrin-labeled anti-IgM mAb (R6–60.2); and FITC-labeled anti-Mac-1 mAb (M1/70), phycoerythrin-labeled anti-Gr-1 mAb (RB6–8C5). Propidium iodide was added to each sample to identify dead cells. The percentages of viable CD4+, CD8+ T cells, B220+ IgM+ B cells, Mac-1+Gr-1+ monocytes, and Mac-1+Gr-1+ granulocytes were distinguished by the laboratory’s FACScan flow cytometer (Becton Dickinson) with analysis performed by using the CELLQUEST software.

Other Colitis-Associated Subphenotypes. On the day of necropsy, feces were assigned a numerical value for consistency (0 = normal, 1 = soft, and 2 = diarrhea). In addition, feces were collected and prepared for secretory IgA measures via sandwich ELISA as described previously (15), with data expressed as ng of IgA/ml. Serum was also collected, and levels of total IgG reactivity against Escherichia coli membrane (ECM) antigens as well as isotype-specific IgG2a were measured by ELISA as described previously (20). Spleens and mesenteric lymph nodes were weighed and expressed as a ratio to body weight.

Statistical Analysis. Genome scans were carried out by using both marker regression and interval mapping analysis. Because similar results were obtained by using both methods, only the results of interval mapping are presented. Logarithm of odds ratio
Distribution of Colitis Lesions. Fig. 1 illustrates the frequency distribution of cecum and colon total scores for the entire F_{2} segregating population (both sexes) compared with the parental and F_{1} strains (n = 408). The parental and F_{1} data shown were reported previously (15) and are reproduced here for comparison to the F_{2} distribution. As reported previously in the F_{1} generation (15), a cluster of colitis-related subphenotypes exhibited positive correlation with the histopathologic colon parameters scored in the segregating F_{2} population. These parameters included spleen/body weight ratio (r = 0.71), mesenteric lymph node/body weight ratio (r = 0.54), and diarrhea grade (r = 0.69). Of the various hematological subphenotypes measured by flow cytometry (percentage of B220^{+} and/or IgM^{+} B cells, CD4^{+} T cells, CD8^{+} T cells, granulocytes, and monocytes), none showed a strong positive or negative correlation with histopathologic scores (data not shown). Neither secretory IgA nor anti-ECM IgG (total) or anti-ECM IgG2a exhibited positive or negative correlations with histopathology scores.

Genetic Linkage Analysis: Main Effects Colitogenic QTL from C3H. Table 1 lists all loci with significant (genome-wide level of 0.01) or suggestive (genome-wide level of 0.05) linkage to colitis histopathology. A highly significant colitogenic QTL contributed by the C3H genome was identified on the distal part of Chr. 3. This locus, provisionally designated Cdcs1, was linked to all the histopathologic parameters except for cecum ulceration and distal colon ulceration. It contributed 15% of the genetic variance for total cecum score and 26% of the variance for total colon score. This QTL was also significantly linked to the colitis-associated subphenotypes of diarrhea and body weight ratio.

Table 2. Significant (LOD ≥4.3) linkage for colitis-related phenotypes detected by genome scans for main effects

<table>
<thead>
<tr>
<th>Marker location</th>
<th>D3Mit348 61.8 cM</th>
<th>D12Nds2 59 cM</th>
<th>D17Nds3* / D17Mit34* 19.06/18.8 cM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen/body weight ratio</td>
<td>15.9</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>Mesenteric lymph node/body weight ratio</td>
<td>18.1</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td>7.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of IgM^{+} B cells</td>
<td>6.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of CD8^{+} T cells</td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Peak LOD score at D17Nds3.

†Peak LOD score at D17Mit34.
IgA and percentage of IgM weight ratio, and the poorly correlated subphenotypes of secretory diarrhea, spleen/body weight ratio, and mesenteric lymph node/D3Mit348 colitis-related subphenotypes.

Fig. 2. Complexity of the broad QTL on Chr. 3 for histopathologic and colitis-related subphenotypes.

Additive and Epistatic Interactions Among Cdcs QTL. Separately, the QTL on Chr. 1, 2, 3, 8, and 17 contributed significant main effects to the histopathologic subphenotypes and other of the colitis-related blood or fecal parameters. When marker alleles for Cdcs1 conferring the major component of colitogenic susceptibility were paired with markers for the other Cdcs QTL with the main effects noted above, additive contributions were noted for most. In addition, pairwise testing across all markers for significant interactions enabled identification of multiple gene epistatic interactions for histopathologic lesions as well as for the other aforementioned phenotypes. The pairs of loci showing strongest evidence for interactions are detailed in Table 3. Two markers on Chr. 18 exhibit their effects as part of interacting pairs with D3Mit348 and D8Mit94. Cdcs1, the major C3H susceptibility QTL on Chr. 3 marked by D3Mit348, contributed

Table 3. Significant pairwise epistatic interactions between marker pairs for various phenotypes

<table>
<thead>
<tr>
<th>Interacting marker pairs</th>
<th>Phenotypes controlled</th>
<th>F overall (&gt;4.7)*</th>
<th>P</th>
<th>F interaction (&gt;4.7)*</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3Mit348 × D18Mit17</td>
<td>Cecum hyperplasia</td>
<td>7.66</td>
<td>1.5 × 10⁻⁹</td>
<td>4.77</td>
<td>9.1 × 10⁻⁴</td>
</tr>
<tr>
<td>D8Mit94 × D18Mit124</td>
<td>Cecum hyperplasia</td>
<td>5.04</td>
<td>5.6 × 10⁻⁶</td>
<td>5.24</td>
<td>4.0 × 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>Cecum total score</td>
<td>5.71</td>
<td>6.8 × 10⁻⁶</td>
<td>6.60</td>
<td>3.8 × 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>anti-ECM IgG</td>
<td>5.49</td>
<td>1.3 × 10⁻⁶</td>
<td>6.11</td>
<td>8.8 × 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>Percentage of IgM⁺ B cells</td>
<td>5.36</td>
<td>2.1 × 10⁻⁶</td>
<td>4.97</td>
<td>6.4 × 10⁻⁴</td>
</tr>
</tbody>
</table>

*F statistic thresholds for significance at genome-wide level of P < 0.01. The genome-wide 0.01 threshold for the simultaneous pairwise genome scan was estimated to be 4.7 by permutation analysis. The test for interaction is based on the nominal 0.001 significance level, which coincidentally is also 4.7.

**Based on tabulated F distribution with 8,399 degrees of freedom.
epistatically with the B6-derived QTL on Chr. 18 (marked by D18Mit7) for the subphenotype cecum hyperplasia. Two B6-contributed colitogenic QTL associated with both cecum hyperplasia and cecum total score were detected by pairwise analysis for epistatic interaction. The Chr. 8 marker was D8Mit94 (13 cM), positioned 8 cM proximal to the Cdcs4 marker (D8Mit191) associated with the main effects. The Chr. 18 marker (D18Mit124 at 32 cM) shown to interact epistatically with the Chr. 8 QTL was positioned 18 cM proximal to the marker shown to interact epistatically with Cdc5 on Chr. 3. Because significant Chr. 18 contributions were detected through epistatic interactions with two different chromosomes (Chr. 3 from C3H and Chr. 8 from B6), a colitogenic QTL on this chromosome was provisionally designated Cdc6.

The detection of additive interaction for mid-colon lesions between D1Mit156, the marker for Cdc32, and D3Mit19 at the distal end of the Cdc3 support interval supported the existence of a C3H-derived colitogenic QTL on Chr. 1. Although no evidence for a significant main effect for loci on Chr. 19 was found, a putative QTL affecting total colon score and middle colon score was indicated through the detection of a significant epistatic interaction between the colitogenic C3H-derived allele at D3Mit257 and a codominant contribution at D19Mit53. Similarly, epistasis was detected with regard to two Ig phenotypes elevated in the C3H.Ii10−/− mutant compared with B6.Ii10−/− parental mice. For anti-ECM IgG and IgM B cell percentage in peripheral blood, the C3H-derived allele at D3Mit189 marking the proximal end of Cdc6 interacted epistatically with the B6-derived D17Mit34 marker for Cdc5. For secretory IgA levels, an epistatic interaction was observed between the B6-derived Cdc4 QTL marked by D8Mit178 and the C3H allele at D12Nds2 (59 cM), a marker within the Ig heavy chain variable chain locus.

**Discussion**

In this study, we located six major QTL contributing to colitis susceptibility as either main effectors and/or interacting QTL. The most significant linkage, provisionally designated Cdc5 on Chr. 3, exhibited C3H susceptibility and was associated with nearly all histopathologic parameters and all colitis-related phenotypes. This locus is the major determinant of colitis susceptibility in this intercross population (accounting for 15–30% of total variance in subphenotypes). However, the secondary QTL represent statistically and biologically significant contributions to the overall phenotype. In the absence of segregation at the Chr. 3 locus, these loci and their interactions would represent major QTL. IBD and colitis susceptibility are clearly complex traits. Comparable epistatic interactions to those underlying colitis susceptibility in our mouse model have been reported recently for IBD susceptibility in humans (9). Table 4 provides a summary of the mouse linkages along with potential human homologs. Several attractive Cdc5 candidate genes are located on Chr. 3 within the 99% confidence interval (65–75 cM). These candidates include the Egf gene (65.2 cM) encoding the epidermal growth factor. The parental strains are polymorphic at this locus; hence, allelic variants could affect mucosal susceptibility to injury and facilitate repair of inflammatory damage. Indeed, therapeutic effects were reported after systemic epidermal growth factor administration to rats with trinitrobenzene sulfonic acid-elicited colitis (23) as well as to humans with gastrointestinal diseases (24). Possibly, epidermal growth factor generates a barrier that limits mucosal injury or functions as a scavenger of reactive oxygen species. In that regard, up-regulation of the Nfkbi1 gene (68.9 cM), encoding the NF-κB p105 transcription factor protein, represents an early response to inflammatory stress (25, 26) with induction observed in areas of inflammation where large quantities of superoxide ion are present such as in colitis. Given the attractiveness of Nfkbi1 as a Cdc5 candidate gene, comparative analysis of C3H versus B6 NF-κB activation in response to lipopolysaccharide (LPS) or IL-12 may be informative. The human ortholog for the Cdc5 locus, if one existed, would be expected on either Chr. 4q or 1p rather than the Crohn’s disease-associated IBDr1 locus on Chr. 16. However, it is intriguing that the IBDr1 candidate gene recently identified, NOD2, is a gene, the product of which activates NF-κB, making the latter responsive to LPS stimulation (27). The reduced LPS sensitivity of C3H mice is linked to a defect of the Toll-like receptor 4 (Tlr4) on Chr. 4. Although the Tlr4 genetic difference did not manifest as a Cdc5 QTL, it is known that additional defects in other receptors such as the IL-12Rβ subunit can contribute to LPS insensitivity (28, 29). Because C3H CD4+ T cells activated by cecal bacterial antigens produce high IFN-γ levels and these C3H T cells transfer colitis when adoptively transferred into C3H-SCID recipients (16), this strain may have evolved mechanisms for maintaining higher NF-κB levels despite the LPS insensitivity. Indeed, we reported previously that bacterial antigen-stimulated CD4+ T cells from C3H.IL-10-deficient mice secreted much higher levels of IFN-γ and IL-3 than did CD4+ T cells from B6.IL-10-deficient mice (15).

Our finding that, in hybrid combinations, the nominally resistant B6 parental genome contributed at least three Cdc5 linkages to the colitis subphenotypes is particularly relevant in understanding why colitis heritability in outbred human populations is so complex. Multiple intestinal neoplasias (Mins) can develop in response to genetic difference did not manifest as a Cdc1 QTL on Chr. 18. (marked by D18Mit17) for the subphenotype cecum hyperplasia. Two B6-contributed colitogenic QTL associated with both cecum hyperplasia and cecum total score were detected by pairwise analysis for epistatic interaction. The Chr. 8 marker was D8Mit94 (13 cM), positioned 8 cM proximal to the Cdcs4 marker (D8Mit191) associated with the main effects. The Chr. 18 marker (D18Mit124 at 32 cM) shown to interact epistatically with the Chr. 8 QTL was positioned 18 cM proximal to the marker shown to interact epistatically with Cdc5 on Chr. 3. Because significant Chr. 18 contributions were detected through epistatic interactions with two different chromosomes (Chr. 3 from C3H and Chr. 8 from B6), a colitogenic QTL on this chromosome was provisionally designated Cdc6.

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**Table 4. Provisional nomenclature for colitogenic linkages**

<table>
<thead>
<tr>
<th>Locus (Chr.)</th>
<th>Susceptibility donor</th>
<th>Mode of action</th>
<th>Phenotype(s) controlled</th>
<th>Candidate genes</th>
<th>Potential human IBD linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdcs1 (3)</td>
<td>C3H</td>
<td>Additive/epistatic with multiple loci</td>
<td>Cecum/colon lesions and multiple subphenotypes</td>
<td>Fabpi, Egf, Nfkbi1</td>
<td>4q or 1p</td>
</tr>
<tr>
<td>Cdcs2 (1)</td>
<td>C3H</td>
<td>Additive</td>
<td>Distal colon severity and hyperplasia</td>
<td>Casp8, Cd28, Cta4, Icos</td>
<td>2q</td>
</tr>
<tr>
<td>Cdcs3 (2)</td>
<td>C3H</td>
<td>Recessive</td>
<td>Distal colon hyperplasia, severity, ulceration, distal colon score, B cell number</td>
<td>Thbs1, B2m</td>
<td>15q</td>
</tr>
<tr>
<td>Cdcs4 (8)</td>
<td>B6</td>
<td>Epistatic</td>
<td>Cecum hyperplasia</td>
<td>Defb, Defcr, Scc8</td>
<td>8p</td>
</tr>
<tr>
<td>Cdcs5 (17)</td>
<td>B6</td>
<td>Recessive</td>
<td>Proximal colon severity, mid-colon ulceration</td>
<td>H2-Ea, C4/Sp, Tnf</td>
<td>6p</td>
</tr>
<tr>
<td>Cdcs6 (18)</td>
<td>B6</td>
<td>Epistatic</td>
<td>Cecum hyperplasia</td>
<td>Dcc, Apc, Mad2, Mad4</td>
<td>18q or 5q</td>
</tr>
</tbody>
</table>

In this study, we located six major QTL contributing to colitis susceptibility as either main effectors and/or interacting QTL. The most significant linkage, provisionally designated Cdc5 on Chr. 3, exhibited C3H susceptibility and was associated with nearly all histopathologic parameters and all colitis-related phenotypes. This locus is the major determinant of colitis susceptibility in this intercross population (accounting for 15–30% of total variance in subphenotypes). However, the secondary QTL represent statistically and biologically significant contributions to the overall phenotype. In the absence of segregation at the Chr. 3 locus, these loci and their interactions would represent major QTL. IBD and colitis susceptibility are clearly complex traits. Comparable epistatic interactions to those underlying colitis susceptibility in our mouse model have been reported recently for IBD susceptibility in humans (9). Table 4 provides a summary of the mouse linkages along with potential human homologs. Several attractive Cdc5 candidate genes are
controlling responsiveness to carcinogen-induced colorectal carcinoma (Scc7) has been localized to ~71cM on Chr. 3. Hence, the Chr. 3/Chr. 8 joint effect could entail additive effects of susceptibilities contributed by each parental genome. It was unexpected that with a main effect of mid-colon ulceration on Chr. 17 (mid-colon ulceration) was derived from the B6 rather than C3H genome. Activated peritoneal macrophages from C3H/HeJ were reported to secrete more tumor necrosis factor-α than those from B6; this phenotype was associated with polymorphisms in the Ifnα promoter region (35). Significantly higher levels of CD8+ T cells circulating in the peripheral blood of our F2 population was associated also with homozygosity for the C3H alleles at D17Mit34 and D17Nds3. However, the MHC haplotype (H2b) of C3H is associated with deletions of genes in the class I Qa2 cluster required for selection of CD8αα cells in the intestinal epithelial lymphocyte compartment (36). Possibly, the presence of functional B6-derived alleles in the Qa2 region represented the increased cytotoxic contribution of this haplotype (H2β). It should be noted that IL-10 deficiency in the strain 129 background, which also expresses the H2b MHC haplotype, also develops severe colitis (14).

In summary, the complexity shown in this analysis of cytokine deficiency-induced colitis in mice underscores the difficulty in elucidating the complex genetics underlying susceptibility to IBD development in humans. We are developing interval-specific congenic stocks (37) to guide analysis of relevant candidate genes in this mouse model system.

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