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# Impaired Resistance and Enhanced Pathology During Infection with a Noninvasive, Attaching-Effacing Enteric Bacterial Pathogen, *Citrobacter rodentium*, in Mice Lacking IL-12 or IFN- $\gamma$

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Mice infected with *Citrobacter rodentium* represent an excellent model in which to examine immune defenses against an attaching-effacing enteric bacterial pathogen. Colonic tissue from mice infected with *C. rodentium* harbors increased transcripts for IL-12 and IFN- $\gamma$  and displays mucosal pathology compared with uninfected controls. In this study, the role of IL-12 and IFN- $\gamma$  in host defense and mucosal injury during *C. rodentium* infection was examined using gene knockout mice. IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice were significantly more susceptible to mucosal and gut-derived systemic *C. rodentium* infection. In particular, a proportion of IL-12p40<sup>-/-</sup> mice died during infection. Analysis of the gut mucosa of IL-12p40<sup>-/-</sup> mice revealed an influx of CD4<sup>+</sup> T cells and a local IFN- $\gamma$  response. Infected IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice also mounted anti-*Citrobacter* serum and gut-associated IgA responses and strongly expressed inducible NO synthase (iNOS) in mucosal tissue, despite diminished serum nitrite/nitrate levels. However, iNOS does not detectably contribute to host defense against *C. rodentium*, as iNOS<sup>-/-</sup> mice were not more susceptible to infection. However, C57BL/6 mice infected with *C. rodentium* up-regulated expression of the mouse  $\beta$ -defensin (mBD)-1 and mBD-3 in colonic tissue. In contrast, expression of mBD-3, but not mBD-1, was significantly attenuated during infection of IL-12- and IFN- $\gamma$ -deficient mice, suggesting mBD-3 may contribute to host defense. These studies are among the first to examine mechanisms of host resistance to an attaching-effacing pathogen and show an important role for IL-12 and IFN- $\gamma$  in limiting bacterial infection of the colonic epithelium. *The Journal of Immunology*, 2002, 168: 1804–1812.

Bacterial pathogens have evolved numerous strategies to avoid host immune effector mechanisms. Some important enteric bacterial pathogens, such as enteropathogenic (EPEC)<sup>2</sup> and enterohemorrhagic (EHEC) *Escherichia coli*, evade many systemic host defense mechanisms by restricting their colonization to the luminal surface of the gut epithelium. The mechanisms by which these pathogens intimately adhere to human epithelial cells represent the most studied feature of EPEC and EHEC biology (reviewed in Refs. 1 and 2). In adhering to intestinal epithelial cells, these bacteria subvert cellular architecture to produce a characteristic histopathological feature known as the attaching and effacing (A/E) lesion (3). EPEC and EHEC are members of a family of noninvasive enteric bacterial pathogens that use A/E lesion formation to colonize the host intestine. For example, *E. coli*

capable of forming A/E lesions has also been recovered from rabbits, pigs, and dogs with diarrhea (4–6). Similarly, the mouse pathogen *Citrobacter rodentium* causes colitis as a consequence of its ability to colonize murine large intestinal enterocytes via A/E lesion formation (7).

In contrast to the detailed information describing the molecular basis for EPEC and EHEC adherence to epithelial cells (2), very little is known about how the host resolves infections caused by these agents. The absence of small animal models to study these human-specific agents directly has made these studies problematic. For this reason, infection of mice with *C. rodentium* has been used as a surrogate model to study host responses to pathogens dependent upon A/E lesion formation for colonization of the host (8). Importantly, *C. rodentium* possesses both established and putative virulence determinants common to human-specific strains of EPEC and EHEC, including a large pathogenicity island (9) and an immunomodulating toxin (10). Furthermore, the A/E lesion induced by *C. rodentium* is ultrastructurally identical to those formed by EHEC and EPEC in animals and humans (7, 11, 12).

In naturally or experimentally infected susceptible mouse strains, *C. rodentium* infection is associated with colonic crypt hyperplasia, goblet cell depletion, and mucosal erosion (13, 14). Oral infection of mice with live *C. rodentium* or intracolonic inoculation of dead bacteria induces a large infiltrate of CD4<sup>+</sup> cells into the colonic lamina propria, a modest increase in epithelial CD8 cells, and a highly polarized Th1 response (8, 15). Transcripts for the type I cytokines IL-12, TNF- $\alpha$ , and IFN- $\gamma$  are highly expressed in colonic tissue of infected mice (8). The role of these cytokines in host defense and mucosal pathology is unclear. Thus

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<sup>2</sup> Abbreviations used in this paper: EPEC, enteropathogenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; MLN, mesenteric lymph node; A/E, attaching and effacing; iNOS, inducible NO synthase; SFC, spot-forming cell; mBD, mouse  $\beta$ -defensin.

it is not known whether the mucosal hyperplasia is part of the protective immune response or is elicited by the pathogen to change its microenvironment for its own benefit. Likewise, it is not known whether the Th1 response in the mucosa is needed for protective immunity, and, even if it is, there is the mechanistic issue of how a Th1 response in the lamina propria can direct effective antibacterial immunity to an organism living on the epithelial surface.

Therefore, the aim of the present study was to examine the role of IL-12 and IFN- $\gamma$  in host defense, the regulation of antibacterial mechanisms, and mucosal injury in *C. rodentium*-infected mice. The results show that IL-12 and IFN- $\gamma$  contribute to host defense in colonic and systemic tissue but that, paradoxically, mucosal damage is greater in IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice. Furthermore, they implicate a role for epithelial antimicrobial peptides of the  $\beta$ -defensin family as components of the host immune response to infection. This study is the first to implicate a role for IL-12 and IFN- $\gamma$  in immunity against a noninvasive, luminal bacterial pathogen of the colon.

## Materials and Methods

### Mice

Female or male 6- to 8-wk-old C57BL/6J mice were purchased from Harlan Olac (Bicester, U.K.) or Bantin & Kingman Universal (Hull, U.K.). IL-12p40<sup>-/-</sup>, IFN- $\gamma$ <sup>-/-</sup>, and inducible NO synthase (iNOS)<sup>-/-</sup> mice (back-crossed to C57BL/6 background at least 10 times) were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained by homozygous matings under contract at Bantin & Kingman Universal. All mice came from specific pathogen-free colonies. During experimental studies, groups of animals were housed in high-efficiency particulate arresting-filtered individually ventilated cages with free access to sterilized food and water.

### Bacterial strains and oral infection of mice

A nalidixic acid-resistant isolate of *C. rodentium* (formerly *Citrobacter freundii* biotype 4280) was used in these studies. The nalidixic acid resistant phenotype of this strain facilitates enumeration of the number of viable *C. rodentium* present in colonic tissues of experimentally infected mice. DBS255(pCVD438) is a *C. rodentium eae* (intimin) mutant complemented with the *eae* gene from EPEC strain E2348/69 (intimin  $\alpha$ ). This strain, which has been described previously (16), expresses biologically active intimin and is virulent in mice. Bacterial inocula were prepared by culturing bacteria overnight at 37°C in 10 ml of Luria broth containing nalidixic acid (100  $\mu$ g/ml) plus chloramphenicol (50  $\mu$ g/ml). Cultures were harvested by centrifugation and resuspended in a one-tenth volume of PBS. Mice were orally inoculated with 200  $\mu$ l of the bacterial suspension using a gavage needle. The viable count of the inoculum was determined by retrospective plating on Luria-Bertani agar containing appropriate antibiotics. In all experiments, mice received 1–4  $\times$  10<sup>9</sup> CFU.

### Recombinant proteins

EspA was cloned from EPEC strain E2348/69, expressed as His-tagged fusion protein in *E. coli* and purified by nickel affinity chromatography as previously described (16, 17).

### Measurement of pathogen burden

At selected time points postinfection, mice were killed by cardiac exsanguination under terminal anesthesia or by cervical dislocation. The terminal 6 cm of the colon was removed and the colon was weighed after removal of fecal pellets. In some experiments, 1-cm samples of distal colon were removed into 10% buffered formalin or snap-frozen in liquid nitrogen for subsequent immunohistological analysis and cytokine RT-PCR. Spleens and colon were homogenized mechanically using a Seward 80 stomacher (Seward Medical, London, U.K.). The number of viable bacteria in organ homogenates was determined by viable count on Luria-Bertani agar containing nalidixic acid and chloramphenicol. The limit of sensitivity was 10 CFU per organ.

### Analysis of humoral immune responses

At selected times postimmunization, 0.2 ml of blood was collected from the tail vein. The serum was collected and stored at -20°C until it was

analyzed. For analysis of Ag-specific Ab responses, wells of microtiter plates (Maxisorb plates; Nunc, Naperville, IL) were coated overnight at 4°C with 100  $\mu$ l of a bicarbonate solution (pH 9.6) containing rEspA (2.5  $\mu$ g/ml). After washing with PBS containing Tween 20 (0.05% v/v), wells were blocked by addition of 1.5% (w/v) BSA in PBS for 1 h. Plates were then washed twice with PBS/Tween 20 before sera from individual mice were added and serially diluted in PBS/Tween 20 containing 0.2% (w/v) BSA and incubated for 2 h at 37°C. For the determination of IgA or IgG Ab titers, wells were washed with PBS/Tween 20 before addition of 100  $\mu$ l of either an IgG- or IgA-specific HRP conjugate (DAKO, High Wycombe, U.K.) diluted 1/1000 in PBS/Tween 20 containing 0.2% (w/v) BSA for 2 h at 37°C. Finally, after washing with PBS/Tween 20, bound Ab was detected by addition of *o*-phenylenediamine substrate (Sigma, Poole, U.K.) and the A<sub>490</sub> was measured. Titers were determined arbitrarily as the reciprocal of the serum dilution corresponding to an OD of 0.3. The minimum detectable titer was 100.

### Determination of total and EspA-specific IgA in fecal samples

Fecal pellets were collected from mice infected with *C. rodentium* 21 days previously. Pellets were weighed, then sterile PBS containing 0.1% sodium azide was added to obtain a concentration of 100 mg/ml. The pellets were homogenized by continuous vortexing for 10 min. Particulate debris was removed by centrifugation (5 min at 14,000 rpm), and supernatants were collected and stored at -70°C until they were required. Total IgA levels in fecal extracts were determined by ELISA. Briefly, levels of total IgA were measured by coating 96-well ELISA plates with 5  $\mu$ g/ml goat anti-mouse  $\alpha$ -chain-specific Abs (Sigma). The plates were blocked with 1.5% BSA in PBS for 1 h at 37°C. Dilutions of stool supernatants were added to the wells and incubated overnight at 4°C, washed, and incubated with  $\alpha$ -chain-specific biotin-conjugated goat anti-mouse Ab for 2 h at 37°C (Sigma-Aldrich, St. Louis, MO). HRP-conjugated streptavidin was used to detect bound Ab. Total IgA in each sample was determined by comparison with a standard curve generated using mouse myeloma IgA (ICN Pharmaceuticals, Bristol, U.K.). Titers of EspA-specific IgA were determined by coating ELISA plates with 2.5  $\mu$ g/ml rEspA and using the  $\alpha$ -chain-specific biotin-conjugated goat anti-mouse Ab as described in the previous section. The EspA-specific IgA titers thus obtained were divided by the total amount of IgA in the sample to correct for variation in the amount of stool collected.

### Detection of cytokine-specific T cell responses by ELISPOT

Cytokine-specific ELISPOT was performed essentially as described previously (18). Briefly, mesenteric lymph nodes (MLNs) from uninfected and infected C57BL/6, IL-12p40<sup>-/-</sup>, and IFN- $\gamma$ <sup>-/-</sup> mice ( $n = 4$ ) were aseptically removed and single cell suspensions were prepared by passing tissue through 100- $\mu$ m nylon sieves (Marathon Laboratories, London, U.K.). Cells were washed once with RPMI 1640 (Sigma) then resuspended in RPMI 1640 containing 10% FCS (Sigma), 5  $\times$  10<sup>-5</sup> M 2-ME, 2 mM L-glutamine (Sigma), 100 U/ml penicillin (Sigma), and 100  $\mu$ g/ml streptomycin (Sigma). Graded numbers of cells were seeded into wells of nitrocellulose-based 96-well microtiter plates (Multiscreen-HA; Millipore, Bedford, MA), which had previously been coated overnight at 4°C with 50  $\mu$ l/well either anti-IFN- $\gamma$  (4  $\mu$ g/ml, clone R46A2) or anti-IL-4 (4  $\mu$ g/ml, clone 11B11) mAb diluted in carbonate buffer (pH 9.6). All Abs were from BD Pharmingen (Cowley, U.K.). Cells were incubated for 20–24 h at 37°C in 5% CO<sub>2</sub>. Following incubation, cells were removed by washing three times for 5 min with PBS, followed by an additional three times with PBS/Tween 20 (0.05% v/v), then 50  $\mu$ l of the biotinylated anti-IFN- $\gamma$  (clone XMG1.2) or anti-IL-4 (clone BVD6-24G2) Abs (1  $\mu$ g/ml in filtered PBS/Tween 20) was added to each well for 2 h. After washing plates five times with filtered PBS/Tween 20, a 1/1000 dilution of extravidin-alkaline phosphatase (Sigma) was added to all wells for 1–2 h at room temperature. Finally, after washing three times with PBS/Tween 20 and once with PBS alone, a solution of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Fast BCIP/NBT; Sigma) was added as substrate. Spots representing single IFN- $\gamma$ - or IL-4-producing cells were counted using a dissecting microscope.

### RNA extraction and quantitative RT-PCR

All molecular biology grade reagents were purchased from Life Technologies (Paisley, U.K.) or Promega (Southampton, U.K.). Total cellular RNA was isolated from frozen colonic tissue by homogenization of the tissue in TRIzol followed by CHCl<sub>3</sub> extraction and isopropanol precipitation. Total RNA was measured by spectrophotometric analysis. Constructs encoding standard RNAs (pCMQ1, pCMQ2, pCMQ3, and pCMQ4), kindly provided by M. F. Kagnoff (Department of Medicine, University of California, San Diego, CA), were used for quantitative competitive RT-PCR. To generate standard RNA, plasmids were linearized with *Sall* (pCMQ1) and *NotI*

(pCMQ2, -3, and -4) and transcribed in vitro with T7 RNA polymerase under conditions recommended by the supplier (Promega). Serial 10-fold dilutions of standard RNA (1 pg to 1 fg) were co-reverse transcribed with total cellular RNA (1  $\mu$ g) at 42°C for 50 min in a 20- $\mu$ l reaction volume containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 3 mM DTT, 10 mM dNTP mix, and 0.5  $\mu$ g of oligo(dT) (Pharmacia Biotech, St. Albans, Hertfordshire, U.K.), using 100 U of reverse transcriptase (Superscript II; RNase H-negative). PCR amplification was routinely conducted in a 50- $\mu$ l reaction volume (10 mM Tris (pH 9), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each dNTP, and 20 pmol specific 5' and 3' primers), using 1 U of *Taq* polymerase (Pharmacia Biotech). The temperature profile of the amplification consisted of 35 cycles of 45 s denaturation at 94°C, 75 s annealing at 58°C, and 75 s extension at 72°C. PCR products were then separated on a 1% agarose gel and bands intensities were quantified by densitometry (Seescan, Cambridge, U.K.). The above protocol allows quantitation to 10<sup>3</sup> cytokine mRNA transcripts per microgram of total RNA.

#### Measurement of mBD mRNA expression by semiquantitative RT-PCR

The mRNA expression of constitutive mouse  $\beta$ -defensin (mBD)-1 and inducible (mBD-3)  $\beta$ -defensins was measured by semiquantitative RT-PCR. The primers used in the study were as follows: mBD-1 sense (5'-<sup>30</sup>ggctccaccac tatgaaac<sup>57-3'</sup>) and antisense (5'-<sup>177</sup>gagacagaatccctcatgtgaa<sup>151</sup>), and mBD-3 sense (5'-<sup>54</sup>gcatttgaggaaaggaactccacaac<sup>77-3'</sup>) and antisense (5'-<sup>186</sup>gtctccacct gcagcttttagca<sup>161-3'</sup>). PCR conditions were optimized and the mRNA expression was measured during the linear phase of the reaction. The PCR amplification cycle was as follows: 90 s at 94°C, 90 s at 58°C, and 90 s at 72°C for 35 cycles. The PCR products were fractionated on a 2.5% agarose and their intensity was measured by densitometry as described above. The PCR product sizes were 147 bp (mBD-1) and 132 bp (mBD-3). The results are presented as a ratio of defensin intensity over GAPDH (housekeeping gene) intensity.

#### Immunohistochemistry and measurement of crypt lengths

Three-step avidin-peroxidase staining was performed on 5- $\mu$ m frozen sections as described previously (8) using mAbs 145-2C11 (anti-CD3), YTS191 (anti-CD4), and YTS169 (anti-CD8). The anti-iNOS Ab was an affinity-purified rabbit IgG polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA). Biotin-conjugated rabbit anti-rat IgG, goat anti-rabbit (DAKO), or goat anti-hamster IgG (Vector Laboratories, Peterborough, U.K.) were used at 1/50 dilution in TBS (pH 7.6) containing 4% (v/v) normal mouse serum (Harlan Seralab, Oxon, U.K.). Avidin-peroxidase (Sigma) was used at a dilution of 1/200 in TBS. A two-step protocol was performed with rabbit anti-intimin Ab (8) together with HRP-conjugated swine anti-rabbit IgG secondary Ab. Peroxidase activity was detected with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 0.5 mg/ml Tris-HCl (pH 7.6), containing 0.01% H<sub>2</sub>O<sub>2</sub> (Sigma). The density of positive cells in the lamina propria was determined by image analysis. Crypt length was measured by micrometry on H&E-stained sections, with 10 measurements being taken in the distal colons of individual mice. Only well-orientated crypts were counted.

#### Measurement of nitrate/nitrite levels in sera

Serum NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels were measured in individual mice using a nitrate/nitrite colorimetric assay in accordance with the manufacturer's instructions (R&D Systems, Abingdon, U.K.). As recommended, sera were passed through a 10-kDa molecular mass filter (Microcon 10; Millipore, Bedford MA) before use in the assay.

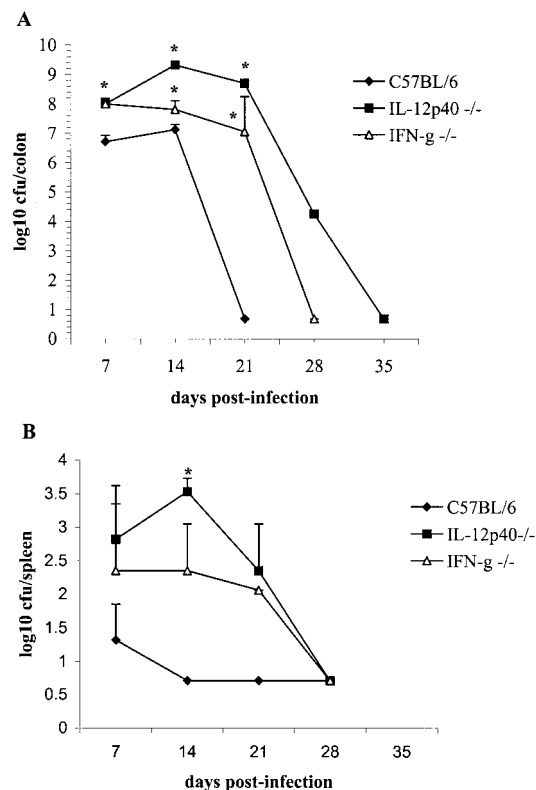
## Results

### Mice deficient in the type I cytokines IL-12 and IFN- $\gamma$ are more susceptible to oral *C. rodentium* infection

Mice infected with *C. rodentium* develop colitis and mount a highly polarized gut Th1 response (8). A component of the response in colonic tissue of infected mice includes increased transcripts for IL-12, IFN- $\gamma$ , IL-1, and TNF- $\alpha$  (8). To determine whether the elevated transcripts for IL-12 and IFN- $\gamma$  contribute to host defense, mice with targeted mutations in the IL-12p40 subunit or IFN- $\gamma$  gene were orally infected with *C. rodentium* strain DBS255(pCVD438). At various times postinfection, mice were

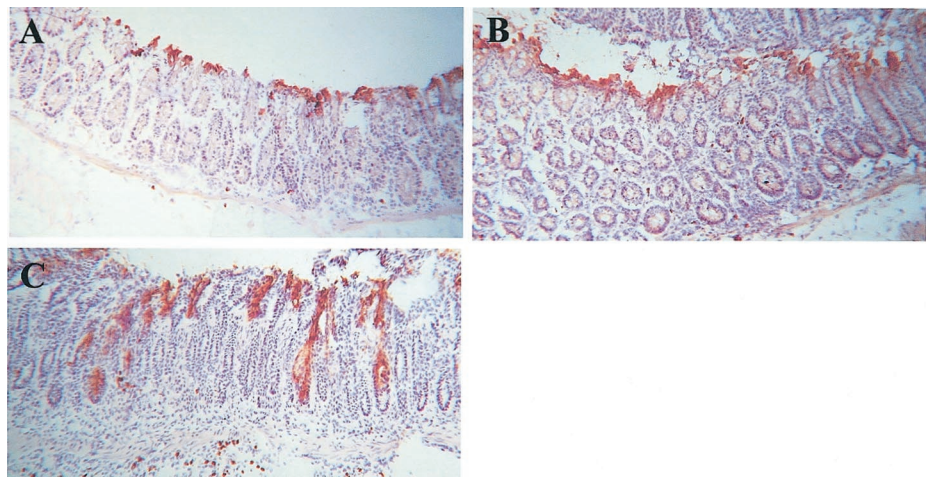
killed and tissue was homogenized for determination of pathogen burden. Infected IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice had significantly higher numbers of *C. rodentium* in the colon on days 7, 14, and 21, and in the spleen on day 14 compared with C57BL/6 mice (Fig. 1, A and B). In addition, there were significantly more *C. rodentium* shed in the stool of IL-12p40<sup>-/-</sup> mice (data not shown). Like C57BL/6 mice, all IFN- $\gamma$ <sup>-/-</sup> mice resolved the infection, although the time to clearance was delayed (Fig. 1A). In contrast, in three separate experiments, 10–15% of IL-12p40<sup>-/-</sup> mice succumbed to infection between days 14 and 21. On the few occasions when tissue from moribund IL-12p40<sup>-/-</sup> mice could be collected, substantial numbers (>10<sup>4</sup> CFU/organ) of *C. rodentium* were recovered from the spleen and liver (data not shown), suggesting mortality was associated with systemic bacteremia. IL-12p40<sup>-/-</sup> mice that did not die between days 14 and 21 cleared the infection by day 35, 2 wk later than C57BL/6 control animals and marginally later than IFN- $\gamma$ <sup>-/-</sup> mice (Fig. 1A).

In addition to the gross microbiological differences between infected C57BL/6 and IL-12p40<sup>-/-</sup> mice, subtle differences in the mucosal location of the infecting bacteria were also observed. Immunostaining of *C. rodentium* in colonic tissue from C57BL/6 mice and IFN- $\gamma$ <sup>-/-</sup> mice showed that most of the adherent bacteria were restricted to the mucosal surface epithelium (Fig. 2, A and B). Specific immunostaining for *C. rodentium* in IL-12p40<sup>-/-</sup> (Fig. 2C) mice revealed adherent bacteria similarly on the mucosal surface, but organisms were occasionally seen penetrating deeper into the crypts.



**FIGURE 1.** IL-12p40- and IFN- $\gamma$ -deficient mice are more susceptible to *C. rodentium* infection than are isogenic C57BL/6 mice. The data depict the mean number ( $\pm$  SD) of DBS255(pCVD438) recovered from colons (A) and spleens (B) of mice orally infected with  $2\text{--}3 \times 10^9$  CFU of DBS255(pCVD438). There was significantly more *C. rodentium* recovered from tissue of IL-12- and IFN- $\gamma$ -deficient mice relative to C57BL/6 mice at the indicated time points (\*,  $p < 0.05$ ). The data are pooled from two separate experiments. The detection limit was 10 CFU/organ.

**FIGURE 2.** Immunohistochemistry of colonic tissue from infected wild-type and knockout mice. Colonic tissue was removed from the distal colon of C57BL/6, IL-12p40<sup>-/-</sup>, and IFN- $\gamma$ <sup>-/-</sup> mice 14 days after oral infection with *C. rodentium*. Tissue sections were stained with an Ab specific for the *C. rodentium* outer membrane protein, intimin. The images ( $\times 100$  magnification) depict representative sections of immunostained colonic tissue from orally infected C57BL/6 mice (A), orally infected IFN- $\gamma$ <sup>-/-</sup> mice (B), and orally infected IL-12p40<sup>-/-</sup> mice (C). Staining for *C. rodentium* was completely absent in tissue from uninfected mice (data not shown). The illustration clearly shows that in IL-12<sup>-/-</sup> mice the bacteria colonize the glands.

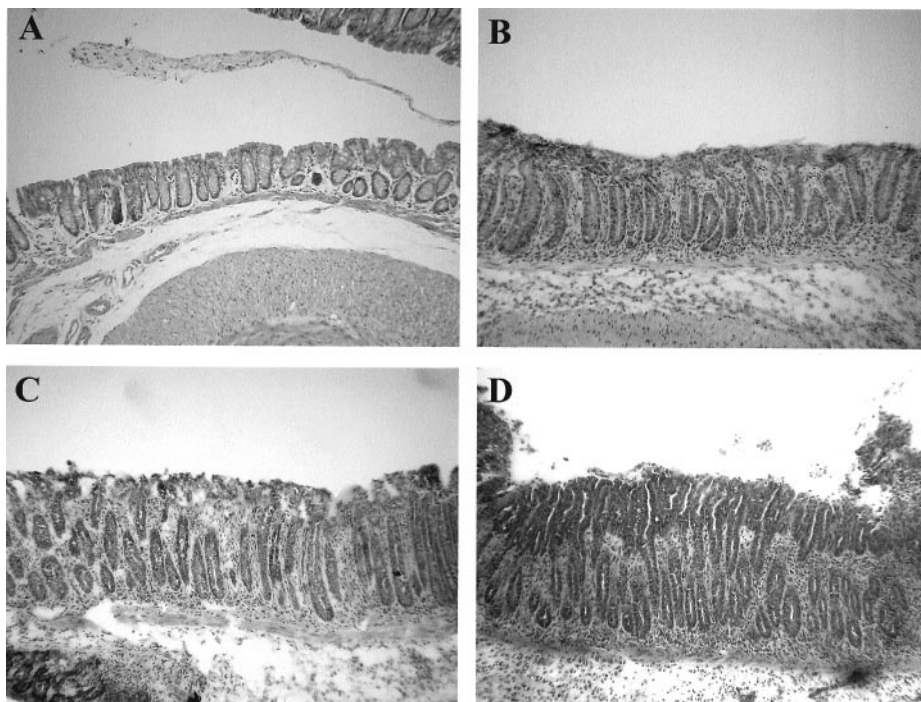


#### Increased colonic pathology in infected IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice

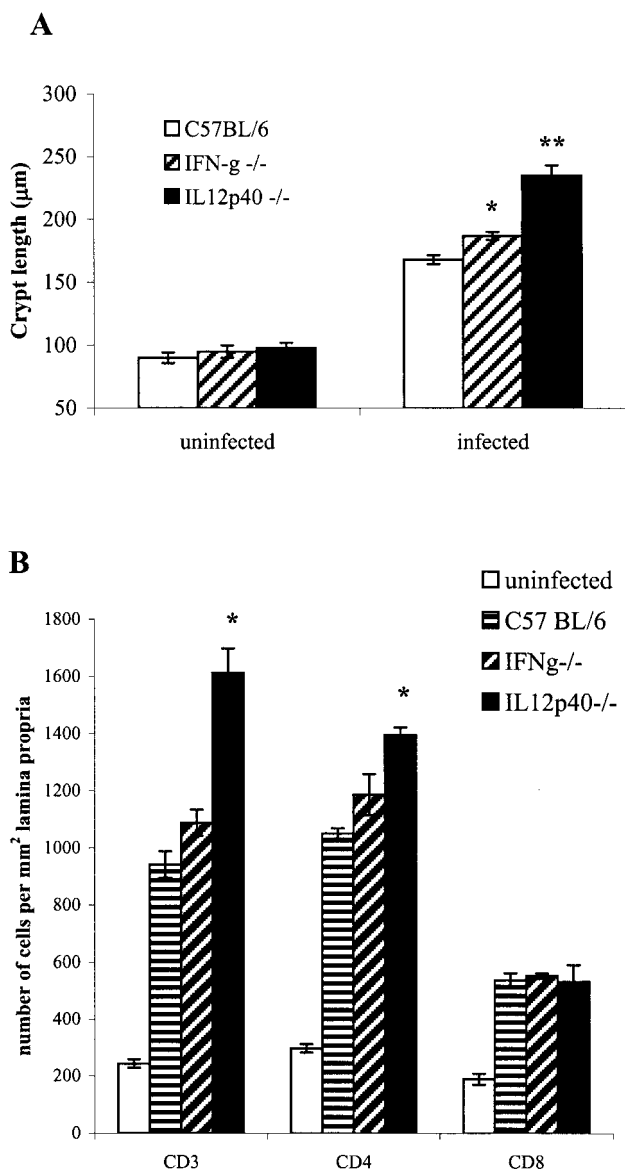
Coincident with the increased pathogen burden in IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice was the development of more dramatic pathological changes in the distal colon. Infected IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice had more crypt hyperplasia than C57BL/6 mice 14 days after infection, although the effect was much greater in the IL-12<sup>-/-</sup> mice than the IFN- $\gamma$ <sup>-/-</sup> mice (Fig. 3). In IL-12p40<sup>-/-</sup> mice in particular, there were focal crypt abscesses and mucosal erosion. It was noticeable at early time points that whereas the enhanced hyperplasia in IFN- $\gamma$ <sup>-/-</sup> mice was quite uniform throughout the colon, there was considerable heterogeneity in the IL-12p40<sup>-/-</sup> mice. Some regions of the mucosa were markedly hyperplastic and there was epithelial shedding; however, in others the mucosa was only slightly thickened. In addition, compared with uninfected mice, there was some loss of goblet cells in infected wild-type animals. However, in infected IFN- $\gamma$ <sup>-/-</sup> mice or IL-12<sup>-/-</sup> mice, loss of goblet cells was much more dramatic (data not shown). The

magnitude of mucosal thickening in infected mice was determined by measurement of crypt length. Compared with infected C57BL/6 mice, crypt lengths were significantly increased in IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice (Fig. 4A). Colons of IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice also weighed significantly more per unit length than those from C57BL/6 mice 14 and 28 days postinfection (data not shown). The colonic lamina propria of infected wild-type mice contained significantly more CD4 T cells than uninfected mice (Fig. 4B). Although there was a modest statistically insignificant increase in CD4 cells in the lamina propria of IFN- $\gamma$ <sup>-/-</sup> mice, in infected IL-12p40<sup>-/-</sup> mice there were significantly more CD4<sup>+</sup> T cells in the lamina propria than in infected IFN- $\gamma$ <sup>-/-</sup> or C57BL/6 mice (Fig. 4B).

CD3<sup>+</sup> intraepithelial lymphocytes were rare in uninfected C57BL/6 mice (1.4 of 100 epithelial cells). In infected C57BL/6 mice there was a modest increase to 3.7 of 100 epithelial cells and virtually identical increases were seen in IL-12<sup>-/-</sup> mice and IFN- $\gamma$ <sup>-/-</sup> mice (data not shown).



**FIGURE 3.** Pathology in colonic tissue of infected mice. Colonic tissue was removed from the distal colon of C57BL/6, IL-12p40<sup>-/-</sup>, and IFN- $\gamma$ <sup>-/-</sup> mice 14 days after oral infection with *C. rodentium*, and frozen sections were cut and stained with H&E. The images ( $\times 100$  magnification) depict tissue from uninfected C57BL/6 mice (A), infected C57BL/6 mice (B), infected IFN- $\gamma$ <sup>-/-</sup> mice (C), and infected IL-12<sup>-/-</sup> mice (D). Infection clearly caused mucosal thickening in wild-type mice. This was somewhat enhanced in IFN- $\gamma$ <sup>-/-</sup> mice but was dramatically enhanced in IL-12<sup>-/-</sup> mice.

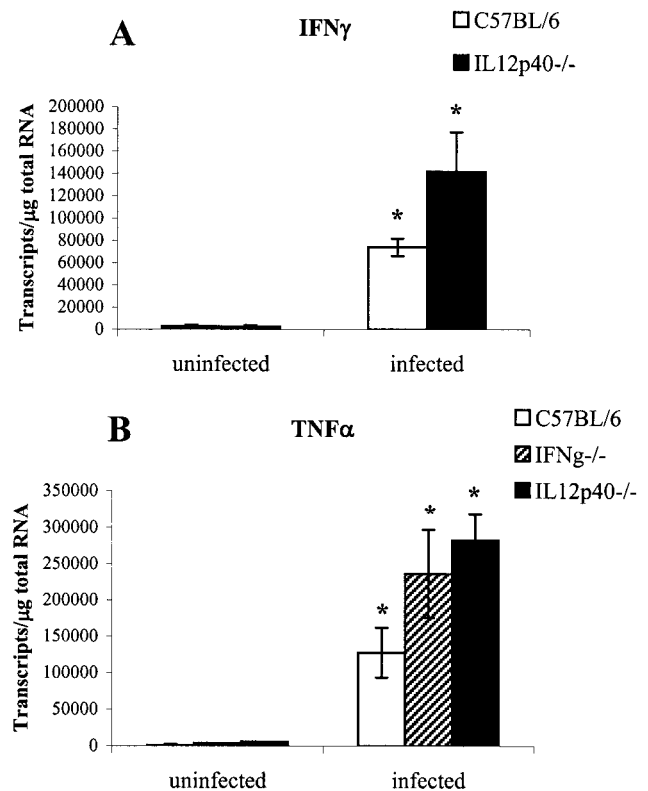


**FIGURE 4.** Mucosal thickness and cellular infiltration in infected IL-12p40<sup>-/-</sup> mice and IFN- $\gamma$ <sup>-/-</sup> mice. **A**, Average colonic crypt length ( $\pm$  SD) in uninfected and *C. rodentium*-infected C57BL/6, IL-12p40<sup>-/-</sup>, and IFN- $\gamma$ <sup>-/-</sup> mice 14 days postinfection. Average crypt length was significantly greater in infected C57BL/6 and IL-12- and IFN- $\gamma$ -deficient mice compared with uninfected mice ( $p < 0.05$ , Student's *t* test). In addition, crypt length was greater in infected IFN- $\gamma$ <sup>-/-</sup> mice (\*,  $p < 0.05$ ) and IL-12<sup>-/-</sup> mice (\*\*,  $p < 0.01$ ) than infected C57BL/6 mice. **B**, The mixed cellular infiltrate in the lamina propria of infected mice include T cells. The data depict the mean number of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells present in the lamina propria of mice infected 14 days previously. There were significantly more CD3<sup>+</sup>, CD4<sup>+</sup>, or CD8<sup>+</sup> T cells in infected wild-type mice compared with uninfected mice ( $p < 0.05$ , Student's *t* test). The lamina propria of infected IL-12p40<sup>-/-</sup> mice also contained significantly more CD3<sup>+</sup> and CD4<sup>+</sup> T cells than infected IFN- $\gamma$ <sup>-/-</sup> or infected C57BL/6 mice (\*,  $p < 0.05$ ).

#### IFN- $\gamma$ expression in mucosal and lymphoid tissues of IL-12p40<sup>-/-</sup> mice infected with *C. rodentium*

IL-12 and IFN- $\gamma$  are, indirectly and directly, important activators of antimicrobial mechanisms in phagocytes. Therefore, a basis for the susceptibility of IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice to *C. rodentium* infection may be an inability to optimally activate antimicrobial mechanisms in phagocytes that neighbor colonic epithelial cells

due to either attenuated (IL-12p40<sup>-/-</sup>) or absent (IFN- $\gamma$ <sup>-/-</sup>) IFN- $\gamma$  expression. To determine whether IFN- $\gamma$  expression was indeed reduced in infected IL-12p40<sup>-/-</sup> mice, IFN- $\gamma$  transcripts in colonic tissue were measured in infected C57BL/6 and IL-12p40<sup>-/-</sup> mice by RT-PCR. IFN- $\gamma$  transcripts were increased in colonic tissue of infected wild-type and IL-12p40<sup>-/-</sup> mice compared with uninfected C57BL/6 mice (Fig. 5A). TNF transcripts were also significantly more abundant in colonic tissue of infected wild-type, IL-12p40<sup>-/-</sup>, and IFN- $\gamma$ <sup>-/-</sup> mice than in infected C57BL/6 mice (Fig. 5B). In addition, there were significantly more TNF transcripts in the colon of infected IL-12<sup>-/-</sup> mice than in infected C57BL/6 mice. IL-4 transcripts were very low and similar in colonic tissues of all infected mouse strains (data not shown). The number of IFN- $\gamma$ -secreting cells were also measured in the MLNs of uninfected and infected mice by direct ex vivo ELISPOT. First, there was no difference in the mean number of IFN- $\gamma$  spot-forming cells (SFCs) detected in uninfected IL-12p40<sup>-/-</sup> mice (22  $\pm$  11) vs uninfected C57BL/6 mice (21  $\pm$  10). However, the mean number of IFN- $\gamma$  SFCs in the MLNs of infected IL-12p40<sup>-/-</sup> mice (29  $\pm$  20) was significantly lower ( $p < 0.05$ ) than in the lymph nodes of infected C57BL/6 mice (82  $\pm$



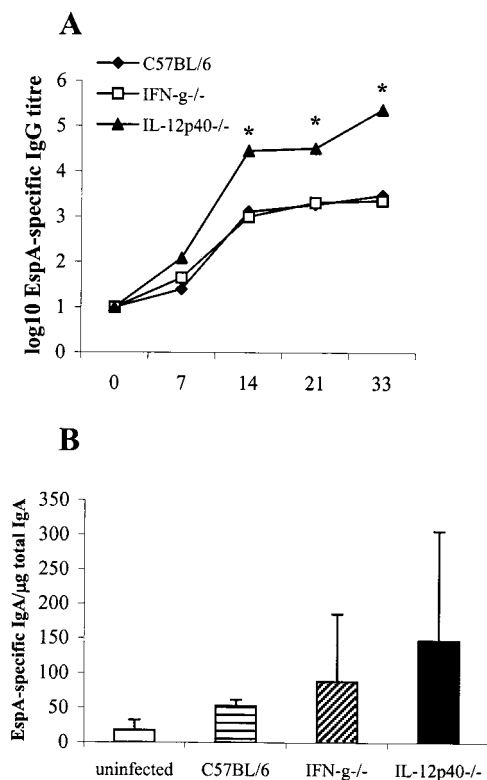
**FIGURE 5.** IL-12-independent IFN- $\gamma$  expression in colonic tissue of infected IL-12p40<sup>-/-</sup> deficient mice. Total RNA was isolated from colonic tissue of mice orally infected with *C. rodentium* 14 days previously. **A**, The data depict the mean number of transcripts ( $\pm$  SEM) encoding IFN- $\gamma$  in infected and uninfected C57BL/6 and IL-12p40<sup>-/-</sup> mice. There were significantly more transcripts detected in infected mice compared with uninfected controls (\*,  $p < 0.01$  for both groups). There was no significant difference in levels of IFN- $\gamma$  transcripts between infected C57BL/6 and IL-12p40<sup>-/-</sup> mice. IFN- $\gamma$  transcripts were undetectable in IFN- $\gamma$ <sup>-/-</sup> mice. **B**, The data depict the mean number of transcripts ( $\pm$  SEM) encoding TNF in infected C57BL/6, IL-12p40<sup>-/-</sup>, and IFN- $\gamma$ <sup>-/-</sup> mice. There were significantly more TNF transcripts in tissue of all infected mice compared with their respective uninfected controls (\*,  $p < 0.05$ ). TNF transcripts were also more abundant in infected IL-12p40<sup>-/-</sup> mice compared with infected C57BL/6 mice (\*\*,  $p < 0.05$ ).

30). There were no significant differences in the number of IL-4 SFCs detected in lymph nodes of the two mouse strains (data not shown). Although IFN- $\gamma$  responses were measured by two different methods, these data may suggest that an IFN- $\gamma$  response can occur in the absence of IL-12 in infected colonic mucosa, but in lymph nodes draining the site of infection the IFN- $\gamma$  response is more IL-12 dependent.

#### Humoral immune responses to EspA in infected mice

To determine whether systemic or intestinal Ab responses to *C. rodentium* were attenuated in the absence of IL-12 or IFN- $\gamma$ , Ig responses were measured against a whole-cell lysate of *C. rodentium* and rEspA, an immunogenic and important virulence determinant in attaching-effacing pathogens (19). EspA-specific serum IgG responses in infected IL-12p40 $^{-/-}$  and IFN- $\gamma^{-/-}$  mice were as robust as those in infected C57BL/6 mice (Fig. 6A). Indeed, EspA-specific responses in IL-12p40 $^{-/-}$  mice were greater than in IFN- $\gamma^{-/-}$  and C57BL/6 mice. An almost identical IgG response pattern was observed when a whole-cell lysate of *C. rodentium* was used as Ag (data not shown).

IFN- $\gamma$  is a central regulator of polymeric IgR expression in intestinal epithelial cells (20). The polymeric IgR mediates the transport of polymeric Ig, predominantly dimeric IgA, from the basolateral to the apical surface of mucosal epithelia. Total and EspA-specific IgA in fecal extracts from infected IL-12p40 $^{-/-}$  and IFN- $\gamma^{-/-}$  mice were, however, not significantly different from those of C57BL/6 mice (Fig.

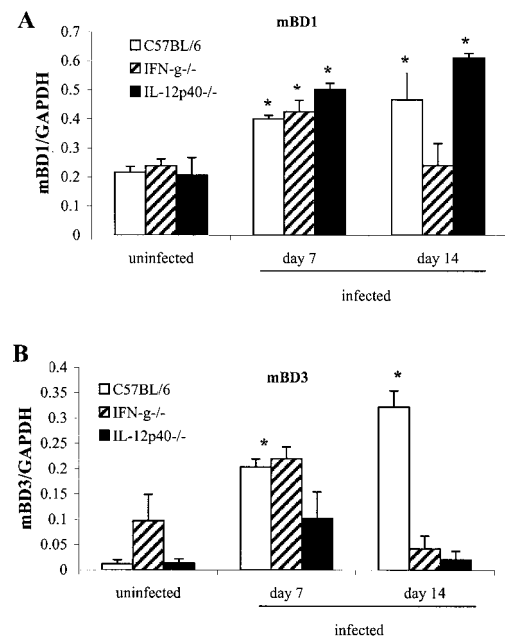


**FIGURE 6.** Systemic and mucosally associated Ig responses to *C. rodentium* EspA are not attenuated in infected IL-12- or IFN- $\gamma$ -deficient mice. *A*, The data depict serum IgG responses to EspA in infected C57BL/6, IL-12p40 $^{-/-}$ , and IFN- $\gamma$ -deficient mice. The EspA-specific titer was significantly greater in IL-12p40 $^{-/-}$  mice than in other groups on days 14, 21, and 33 (\*,  $p < 0.05$ ). *B*, The data depict the mean anti-EspA IgA Ab titer detected in stool samples from infected mice 21 days after infection. Mucosa-associated EspA-specific IgA responses were not significantly different between infected C57BL/6, IL-12p40 $^{-/-}$ , and IFN- $\gamma$ -deficient mice.

6B). Collectively, these data suggest that the basis for the enhanced susceptibility of IL-12p40 $^{-/-}$  and IFN- $\gamma^{-/-}$  mice does not lie in defective systemic or gut-associated IgA responses.

#### Expression of $\beta$ -defensins in colonic tissue of *C. rodentium*-infected mice

$\beta$ -defensins are a family of small cationic antimicrobial peptides, which appear to be widely expressed throughout the gut. Previous studies have suggested that the expression of some  $\beta$ -defensins are up-regulated by proinflammatory cytokines (21). The expression of two  $\beta$ -defensins, mBD-1 and mBD-3, was therefore monitored in colonic tissue to determine whether  $\beta$ -defensins were induced during *C. rodentium* infection of C57BL/6 mice and whether their expression was affected in mice deficient in IL-12 or IFN- $\gamma$ . By RT-PCR, transcripts encoding mBD-1, and to a much lesser extent mBD-3, were detected in colonic tissue of uninfected C57BL/6, IL-12p40 $^{-/-}$ , and IFN- $\gamma^{-/-}$  mice (Fig. 7, *A* and *B*). In mice orally infected with *C. rodentium*, transcripts encoding mBD-1 were increased compared with the relevant uninfected mice on day 7. For wild-type and IL-12 $^{-/-}$  mice mBD-1 transcripts were still elevated on day 14 (Fig. 7*A*). In C57BL/6 mice, transcripts encoding mBD-3 were also highly induced by infection. However, expression of mBD-3 in IL-12p40 $^{-/-}$  and IFN- $\gamma^{-/-}$  mice was not induced to the same degree as occurred in C57BL/6 mice, particularly 14 days postinfection (Fig. 7*B*). Indeed, whereas mBD-3 transcripts increased in C57BL/6 mice between days 7 and 14, in IL-12p40 $^{-/-}$  and IFN- $\gamma^{-/-}$  mice transcripts actually fell (Fig. 7*B*). This occurred despite IL-12p40 $^{-/-}$  and IFN- $\gamma^{-/-}$  mice having a greater pathogen burden at this time point. Upon clearance of



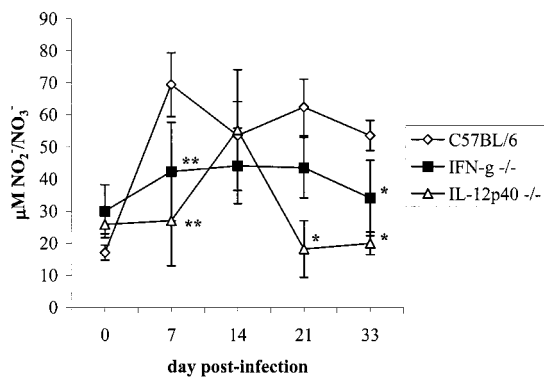
**FIGURE 7.** Expression of  $\beta$ -defensins in colonic tissue of mice infected with *C. rodentium*. The expression of GAPDH and two  $\beta$ -defensins, mBD-1 and mBD-3, was monitored in colonic tissue of infected C57BL/6, IFN- $\gamma^{-/-}$ , and IL-12p40 $^{-/-}$  mice by RT-PCR. *A*, Transcripts encoding mBD-1 were present in tissue of uninfected mice and were significantly up-regulated during infection with *C. rodentium* on day 7 (\*,  $p < 0.05$  vs relevant uninfected group). In C57BL/6 mice and IL-12 $^{-/-}$  mice, transcripts were still raised on day 14. *B*, Expression of mBD-3 was induced during infection of C57BL/6 mice (\*,  $p < 0.05$  vs uninfected C57BL/6 mice on days 7 and 14). In IL-12p40 $^{-/-}$  and IFN- $\gamma^{-/-}$  mice there was no increase in transcripts on days 7 and 14 of infection and on day 14 transcripts were significantly less than in infected C57BL/6 mice (\*,  $p < 0.05$ ).

*C. rodentium* from tissues of C57BL/6 mice, the expression of mBD-3 returned to preinfection levels (data not shown). These data show that mBD-3 expression is attenuated in infected IL-12- and IFN- $\gamma$ -deficient mice. Potentially, the attenuated expression of mBD-3 in the colons of these animals might contribute to their enhanced susceptibility to *C. rodentium* infection.

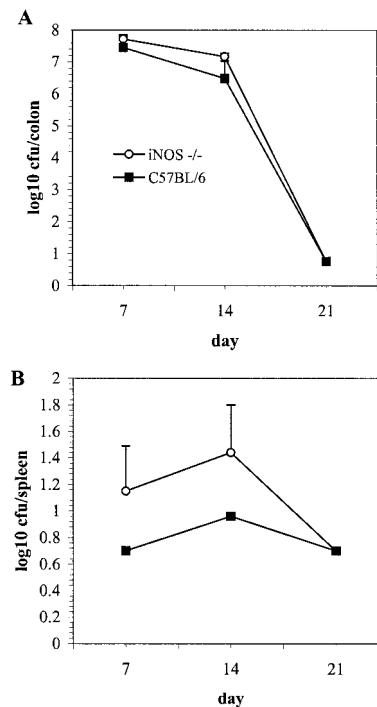
#### Reduced $\text{NO}_3^-/\text{NO}_2^-$ levels in infected IL-12- and IFN- $\gamma$ -deficient mice

Type 2 iNOS contributes to antimicrobial effector functions in host cells by producing NO, which reacts with oxygen radicals to yield reactive nitrogen intermediates with antimicrobial activity. The stable end products of reactive nitrogen intermediates, nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ), can be detected in ex vivo biological fluids of mice, thereby providing a convenient means to monitor iNOS activity. C57BL/6 mice infected with *C. rodentium* had elevated levels of  $\text{NO}_2^-/\text{NO}_3^-$  in sera as early as day 7 postinfection, and these remained elevated until day 35 (Fig. 8). Compared with levels in C57BL/6 mice,  $\text{NO}_2^-/\text{NO}_3^-$  levels in sera of infected IL-12-deficient mice were significantly lower on days 7, 21, and 33 (Fig. 8).  $\text{NO}_2^-/\text{NO}_3^-$  levels in sera of infected IFN- $\gamma^{-/-}$  mice were also significantly lower on day 7 postinfection compared with C57BL/6 mice (Fig. 8). These data suggested iNOS activity was attenuated in IL-12- and IFN- $\gamma$ -deficient mice infected with *C. rodentium*, despite the presence of a greater pathogen load in these animals. However, immunohistochemistry showed that epithelial iNOS was not seen in uninfected mice but was strongly expressed in epithelial cells of all infected wild-type, IL-12 $^{-/-}$ , and IFN- $\gamma^{-/-}$  mice (data not shown).

To clarify the role of iNOS in host defense against *C. rodentium*, iNOS $^{-/-}$  mice were orally infected with *C. rodentium* and the number of challenge bacteria present in selected tissues was determined. C57BL/6 and iNOS $^{-/-}$  mice controlled and cleared *C. rodentium* from mucosal and systemic tissues with similar kinetics (Fig. 9). Furthermore, there were no apparent differences in the extent or type of pathology that occurred in colonic tissues of infected mice (data not shown). These data imply that although iNOS is expressed at sites of *C. rodentium* infection, its absence in an iNOS $^{-/-}$  mouse does not discernibly alter host resistance to *C. rodentium*.



**FIGURE 8.** Serum nitrate/nitrite levels suggest IL-12 and IFN- $\gamma$  contribute to iNOS activity during *C. rodentium* infection. The data depict the mean levels ( $\pm$  SD) of  $\text{NO}_2^-/\text{NO}_3^-$  in sera of infected C57BL/6, IFN- $\gamma^{-/-}$ , and IL-12p40 $^{-/-}$  mice before and during infection with *C. rodentium*.  $\text{NO}_2^-/\text{NO}_3^-$  levels were significantly lower in infected IL-12 $^{-/-}$  mice on days 7, 21, and 33 compared with C57BL/6 mice (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ), and were also significantly lower in IFN- $\gamma^{-/-}$  mice on days 7 and 33 (\*,  $p < 0.05$ ; \*\*,  $p < 0.05$ ).



**FIGURE 9.** iNOS $^{-/-}$  mice have unimpaired resistance to *C. rodentium*. The data depict the mean number ( $\pm$  SD) of DBS255(pCVD438) recovered from colons (A) and spleens (B) of C57BL/6 and iNOS $^{-/-}$  mice orally infected with  $2-3 \times 10^9$  CFU of DBS255(pCVD438). There were no significant differences in the number of *C. rodentium* recovered from tissue of iNOS $^{-/-}$  mice relative to C57BL/6 mice. The data are from one of three separate experiments, which gave similar results.

## Discussion

*C. rodentium* infection of mice is an accessible and relevant animal model in which to study immune defenses against pathogens that colonize via A/E lesion formation. This study describes the importance of IL-12 and IFN- $\gamma$  in immunity to *C. rodentium* and explores some of the antimicrobial mechanisms through which host resistance may be expressed. IL-12-deficient mice were more susceptible to *C. rodentium* infection than either IFN- $\gamma$ -deficient mice or C57BL/6 mice. The enhanced susceptibility of IL-12p40 $^{-/-}$  and IFN- $\gamma^{-/-}$  mice does not appear to be due to an inability to express TNF in colonic tissue, mount anti-*C. rodentium* Ab responses, or express iNOS at sites of infection. Rather, the enhanced susceptibility of IL-12- and IFN- $\gamma$ -deficient mice might, in part, be due to attenuated expression of the inducible  $\beta$ -defensin mBD-3.

In microbiological and immunological terms, the *C. rodentium* mouse model is unique. *C. rodentium* and some species of *Helicobacter* are examples of bacterial pathogens that are capable of establishing relatively persistent, noninvasive infections in the murine gastrointestinal tract. However, *C. rodentium* is distinguished from *Helicobacter* sp. by its ability to colonize the host in the competitive microenvironment of the colon. In addition, the induction of A/E lesions on gastrointestinal mucosal epithelial cells by pathogens like *C. rodentium* (along with EPEC and EHEC) represents a highly specialized pathogen-host interaction. This may require from the host a specific subset of immune effectors to achieve bacterial clearance from the apical surface of the epithelial cell.

IL-12 is a potent immunoregulatory cytokine that is crucially involved in a wide range of infectious diseases. In several experimental models of bacterial, parasitic, viral, and fungal infection,



endogenous IL-12 is required for early control of infection and for generation of Th1 cells and the expression of phagocyte-mediated protective immunity (22). In the context of *C. rodentium* infection, IL-12 transcripts are increased in colonic tissue of infected mice (8). The importance of IL-12 expression was shown here by the marked increase in susceptibility of IL-12p40<sup>-/-</sup> mice to *C. rodentium* infection. A common theme in several bacterial infections is that IL-12 regulates the magnitude of the IFN- $\gamma$  response at the initiation of infection, thus enhancing phagocyte activation, favoring Th1-cell development, and inhibiting Th2 responses. Nevertheless, the basis for the enhanced susceptibility of IL-12p40<sup>-/-</sup> mice to *C. rodentium* clearly has an IFN- $\gamma$ -independent component, as only IL-12p40<sup>-/-</sup>, but not IFN- $\gamma$ <sup>-/-</sup>, mice died during infection. Furthermore, colonic tissue of infected IL-12p40<sup>-/-</sup> mice contained transcripts encoding IFN- $\gamma$ , suggesting IFN- $\gamma$  may not in fact be limiting in the mucosa of these mice and that an IL-12-independent mechanism of IFN- $\gamma$  induction exists. This observation is not unprecedented. Indeed, several reports have described IL-12-independent induction of IFN- $\gamma$  responses in infection models (23–26). Furthermore, some viral infections can drive IFN- $\gamma$  responses independently of both IL-12 and IL-18 (27).

The generation of reactive nitrogen intermediates in phagocytes by iNOS is regarded as an important mechanism by which phagocytosed pathogens are killed (28). However, iNOS expression is not restricted to phagocytes. Increased iNOS expression has been reported in colonic epithelial cells of patients with inflammatory bowel disease or acute *Shigella colitis* (29, 30). Furthermore, in polarized colonic epithelial cells infected with pathogenic *E. coli*, the stable NO end products, nitrate and nitrite, are preferentially located on the apical side, suggesting that epithelial cell-derived NO and its metabolites might affect pathogens on the luminal side of the epithelium (31). However, the role of iNOS in immunity to a bacterial pathogen that resides on the gastrointestinal mucosa has not previously been addressed in vivo. The data presented in this study, using iNOS<sup>-/-</sup> mice, suggest that iNOS is not essential for control or clearance of *C. rodentium*. This is despite the up-regulation of iNOS on the apical surface of enterocytes in areas of infected epithelium in C57BL/6 mice and an increase in nitrate/nitrite levels in sera. Furthermore, neither IL-12 nor IFN- $\gamma$  appears to be essential for iNOS expression by colonic epithelial cells, as abundant iNOS staining was observed in the epithelia of *C. rodentium*-infected IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice. Other inflammatory mediators, like TNF, may act in concert with signals from *C. rodentium* to increase iNOS expression.

Multiple cell types, including neutrophils, macrophages, and epithelia in different anatomical locations express antimicrobial peptides. The defensins are grouped into  $\alpha$ - and  $\beta$ -defensins, based on different interlinking of internal cysteines. In mice, the  $\alpha$ -defensins are predominantly expressed by intestinal Paneth cells, while the  $\beta$ -defensins have been found mainly in epithelial cells throughout the gut (32). Defensins exhibit antimicrobial activity against Gram-positive and Gram-negative bacterial pathogens including *E. coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, and parasites like *Giardia intestinalis*, supporting the contention that these peptides may contribute directly to host defense (33). In this study, transcripts encoding mBD-1 were elevated above basal levels in colonic tissue of mice infected with *C. rodentium*. Unlike mBD-1, however, expression of mBD-3 in C57BL/6 mice appears to be more tightly regulated, as transcripts encoding mBD-3 were more dramatically up-regulated upon *C. rodentium* infection. Although there are scant data on the in vivo expression of  $\beta$ -defensins during infection, these results are consistent with previous in vitro studies showing rapid induction of mBD-3 expression in epithelial cells upon their interaction with enteric pathogens (21). IL-12 and

IFN- $\gamma$ , directly or indirectly, appear to play regulatory roles in the expression of mBD-3. Infected IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice had lower levels of transcripts encoding mBD-3, despite having a dramatically greater pathogen burden. The attenuated mBD-3 response in these mice might at least partially account for their enhanced susceptibility. However, a direct test of this hypothesis is difficult because defensin-specific neutralizing Abs and  $\beta$ -defensin gene-targeted mice are currently unavailable. Nevertheless, given that infection occurs predominantly on the luminal side of the epithelium, it seems likely that pathogen eradication would be assisted by effector mechanisms, such as mBD-1 and mBD-3, which can operate at the epithelial surface.

The mechanisms by which the host resolves infections by attaching-effacing bacterial pathogens, which include the important human pathogens EPEC and EHEC, remain virtually unexplored. The data presented in this study, using *C. rodentium* as a model system, add to our understanding of the type of immune mechanisms required for resolution of infections caused by this class of pathogen. Importantly, however, apart from a small percentage of IL-12p40<sup>-/-</sup> mice, all animals infected with *C. rodentium* in these studies survived and resolved infection. Clearly, then, there are IL-12- and IFN- $\gamma$ -independent immune mechanisms in operation that result in effective inhibition of pathogen adherence and/or killing of attached bacteria, and the identification of these mechanisms is the focus of our current studies.

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