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Critical Role of T Cell-Dependent Serum Antibody, but Not the Gut-Associated Lymphoid Tissue, for Surviving Acute Mucosal Infection with *Citrobacter rodentium*, an Attaching and Effacing Pathogen

Lynn Bry*† and Michael B. Brenner**

*Citrobacter rodentium* uses virulence factors similar to the enteropathogenic *Escherichia coli* to produce attaching and effacing lesions in the distal colon of mice. We used this infection model to determine components of adaptive immunity needed to survive infection. During acute infection, wild-type mice develop breaks across infected epithelial surfaces but resolve infection. Surprisingly, mice markedly deficient in mucosal lymphocyte populations from β integrin deficiency resolve infection, as do CD8α−/− or TCR-δ−/− mice. In contrast, CD4−/− or TCR-β−/− mice develop polymicrobial sepsis and end-organ damage, and succumb during acute infection, despite epithelial damage similar to wild-type mice. B cell-deficient (MuMT−/−) or B and T cell-deficient (recombinase-activating gene 2−/−) mice develop severe pathology in colon and internal organs, and deteriorate rapidly during acute infection. Surviving mice develop robust *Citrobacter*-specific serum IgM responses during acute infection, whereas mice that succumb do not. However, CD4−/− mice receiving serum Igs from infected wild-type mice survive and clear the infection. Our data show that survival of apparently self-limited and luminal mucosal infections requires a systemic T cell-dependent Ab response against bacteria that enter through damaged mucosa. These findings have implications for understanding host defense against mucosal infections, including the pathogenesis of these diseases in immunocompromised populations. *The Journal of Immunology*, 2004, 172: 433–441.

A ttaching and effacing pathogens, such as the enteropathogenic *Escherichia coli* (EPEC), cause a self-limited enteritis in immunocompetent hosts. In contrast, these infections often cause debilitating disease in immunocompromised populations including young animals, infants (1), SIV+ rhesus macaques, and HIV+ patients (2, 3), suggesting a critical role for adaptive immunity in controlling infection.

*Citrobacter rodentium* is an attaching and effacing pathogen of mice that serves as the animal model for these mucosal infections (4). *C. rodentium* uses virulence factors equivalent to the EPEC to produce attaching and effacing lesions in the distal colon of mice (5). As with EPEC, *C. rodentium* infection progresses through three distinct stages: 1) a colonization phase commonly mediated by bacterial adhesins that facilitate binding to epithelial surfaces, 2) an acute or symptomatic phase manifest as diarrhea and development of the host’s inflammatory response, and 3) a convalescent phase during which the host clears infecting organisms from mucosal environments and tissue pathology inflicted from infection resolves. Each stage of disease involves a complicated interplay of bacterial and host factors that determine the severity of disease as well as the host’s ability to clear infection while inflicting minimal damage to infected tissues.

A variety of host factors impact the initial colonization of mucosal pathogens. The low pH of the stomach provides an inhospitable environment to orally introduced pathogens. Glycosylated mucins and secretary IgA add to the protective barrier over epithelial surfaces and frequently provide competing carbohydrate epitopes for bacterial adhesins (6–9). Within the intestine, bile acids, and anti-bacterial factors including lysozyme (10), phospholipases with specificities for bacterial lipids (11), and cryptdins, small cationic peptides related to the neutrophil defensins (12), affect microbial survival. Colonization with commensals also provides protection by limiting available nutrients and ecological niches in which pathogens may gain a foothold (13).

The first stage of infection for mucosal pathogens thus requires penetration of innate defenses with adherence to epithelial surfaces. Following subversion of these defenses, EPEC and related pathogens develop attaching and effacing lesions mediated by intimin, a bacterially associated adhesin, and translocated intimin receptor, the counterreceptor for intimin. A type III secretion system delivers translocated intimin receptor and products of the *esp* genes (extracellularly secreted proteins), including EspA, EspB, and EspD to the host cell, which collectively act to alter signal transduction pathways within eukaryotic cells (14–16). As a result, extraordinarily high numbers of bacteria adhere and proliferate along luminal surfaces to cause symptomatic disease. In weaning-age mice, the colonization phase of *C. rodentium* infection occurs during the first 7–10 days after inoculation. By the onset of symptoms associated with acute infection, the burden of colonic *C. rodentium* frequently exceeds >10⁹ CFU per gram of tissue. Symptomatic disease occurs over the next 2 wk (17),
during which a secretory diarrhea develops secondary to increased chloride secretion through infected, hyperplastic epithelium (18). The infected colonic epithelium thickens 2- to 3-fold, increasing the surface area on which the bacteria proliferate.

With regard to the host immune response that develops during acute infection, in vitro EPEC adhesion models have demonstrated activation of NF-κB in colonized epithelial cells (19) with release of IL-8 (20), and subsequent transmigration of neutrophils across infected epithelium (21). In vivo, the resulting inflammatory response frequently destroys areas of infected epithelium in the small bowel (22). While focal abscesses develop beneath regions densely colonized with C. rodentium (17), infection with this attaching and effacing pathogen generates a predominately lymphocytic infiltrate, characterized by CD4+ T cells situated near the proliferative epithelial crypts (23). The functions of these lymphocytes and their role in disease have not been well defined.

The convalescent phase of C. rodentium infection occurs over the subsequent 4 wk, during which the diarrhea and epithelial hyperplasia resolve (17). Although mucosal and systemic vaccination strategies with intimin have shown an impact on the development and course of disease caused by C. rodentium (24), the role of adaptive immune responses, including cytotoxic and humoral components, have not been clearly defined in colonization and acute or convalescent stages of primary disease.

To identify lymphocyte populations important for control, survival, and resolution of infection, we inoculated C. rodentium into mice possessing regional lymphocyte deficiencies, or defined deficiencies of T and B cell subsets. Surprisingly, we observed that β2 integrins, important for homing of lymphocytes to mucosal locations, were dispensable for survival and clearance of infection. However, defects affecting the early T cell-dependent serum Ab response to C. rodentium proved critical for survival of acute infection.

Materials and Methods

Bacterial strains
C. rodentium strain DBS 100 (ATCC 51459; American Type Culture Collection, Manassas, VA) was provided by Dr. D. Schauer (Department of Civil Engineering, Division of Bioengineering and Environmental Health, Massachusetts Institute of Technology, Cambridge, MA) (25). Bacteria grown on MacConkey agar (MAC; REMEL, Lenexa, KS) or in Luria-Bertani broth. E. coli isolated from our mouse colony were cultured on MAC from fecal samples and identified by API-20E enteric panels (Bio-Merieux, Hazelwood, MO). Ag typing performed on this strain of E. coli demonstrated it to be O-Ag 112 positive and negative for toxins, the eaeA gene, H, Vi, fimbrial, and flagellar Ags (E. coli Reference Laboratory, College Park, PA).

Mouse strains
αβγ-Integrin-deficient C57BL/6 mice were generated in our laboratory by backcrossing αβγ-deficient BALB/c mice (26) to C57BL/6 mice for 11 generations and confirming the presence of C57BL/6 polymorphisms at 14 cM 5' and 10 cM 3' to the Igae locus. Recombinative-activating gene (RAG2)-deficient mice (27) were obtained from Taconic Farms (Germantown, NY), C57BL/6 wild-type mice and βγ integrin- (28), CD4- (29), CD8α- (30), TCIR-β-chain- (31), TCIR-δ-chain- (32), and MuMT-deficient (33) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mouse strains were housed in the same room under specific pathogen-free conditions, exposed to bedding from existing animals housed in the same room, and bred for a minimum of 2 mo before initiating experiments in weanling-age animals. Fecal samples from breeder pairs were plated to MAC to confirm colonization with a commensal E. coli found in our colony. MuMT mice at 19 days of age were analyzed to identify IgA-negative animals. Experimental MuMT-deficient mice were subsequently tested upon sacrifice to confirm continued lack of IgA in serum and feces.

Identification of IgA-negative MuMT-deficient mice
Fecal homogenates were prepared by vortexing 0.1 mg of fecal pellets in PBS plus 0.1% Tween 20 plus 25 μl of protease inhibitor mixture for general use (Sigma-Aldrich, St. Louis, MO). Fifty microliters of suspended material was extracted in an equal volume of 2X Laemml buffer, heated to 95°C for 5 min, and then spun to pellet solid material (34). For serum analyses, 25 μl of whole blood from anesthetized mice was obtained by orbital sinus puncture, allowed to clot, and treated as described. Samples were subjected to SDS-PAGE with control preparations from wild-type C57BL/6 mice, and serial dilutions of mouse IgA myeloma protein (BD PharMingen, San Diego, CA). Proteins were transferred to GeneScreen X polyvinylidene difluoride membrane using a semidry electroblotting apparatus (Bio-Rad, Hercules, CA). Membranes were blocked in 10% soy milk (8th Continent, Minneapolis, MN) plus 5% rabbit serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.1% Tween 20 (Bio-Rad) in PBS for 2 h before incubation with rabbit anti-mouse IgA conjugated to HRP (Zymed Laboratories, San Francisco, CA) diluted 1/25,000 in soy blocking buffer and incubated with blots for 1 h. After incubation, membranes were washed in PBS plus 0.05% Tween 20 three times. HRP-bound Ab was visualized with ECL (Western Lightning; PerkinElmer, Wellesley, MA). MuMT mice demonstrating no IgA reactivity in serum or fecal homogenates were used in experiments or for breeding (<0.1 ng/μg of feces and <0.1 ng/10 μl of serum). Less than 2% of mice in our colony had detectable IgA at 19 days of age, and <10% had detectable levels at 8 wk.

Infection of mice
Twenty-one-day-old mice were fasted for 8 h before oral inoculation with C. rodentium. While fasting, animals had access to water. A stationary-phase broth culture of C. rodentium was pelleted and resuspended in sterile PBS to an OD600 of 1.0, resuspended and suspended in the original volume of PBS or in 1/10 vol to obtain a 10-fold-concentrated culture. A volume of 0.2 ml of the original or concentrated culture was fed to mice through a neonatal gavage needle to deliver ~5 × 108 (low-dose) or 5 × 109 (high-dose) CFU/mouse. Inoculations and all subsequent manipulations were conducted in BL-2 biosafety cabinets. Animals were allowed access to food after inoculation. Each inoculating culture was serially diluted and plated to confirm CFU administered.

Survival studies
Mice were inoculated as described. Fecal CFU were determined at 3, 7, 15, 21, 28, 35, 42, and 48 days after inoculation. Moribund animals, or those showing unalleviated distress, were anesthetized with sevoflurane volatile anesthetic and euthanized. A minimum of eight mice per strain were analyzed in at least two independent experiments for each log level oral inoculum of C. rodentium administered. Log rank and χ2 analyses were conducted in Prism 3.0 to determine median survival time and statistical significance. A value of p < 0.05 was considered significant.

Tissue collection and histology
Control or infected mice were euthanized as described. Samples of whole blood, spleen, liver, mesenteric lymph node, small intestine, and colon were removed under aseptic conditions and placed in 10% formalin in PBS (FS) or in tissue blocks containing OCT and snap frozen. The colon was dissected to the anal canal and removed en bloc. The terminal 0.5 cm, including the anal canal, was removed and placed in FS. The adjacent 0.5-cm piece was used for CFU analysis. Subsequent 1.0-cm segments were fixed in FS or frozen in OCT. FS-fixed tissues were oriented in 1% agarose before paraffin embedding. Sections (5 μm) were deparaffinized and stained with H&E to evaluate tissue pathology, or with the Steiner silver stain to permit visualization of enteric bacteria (35). Steiner silver stains were compared with frozen sections from adjacent tissue stained with polyclonal rabbit anti-Citrobacter intimin sera kindly provided by Dr. G. Frankel (Centre for Molecular Microbiology and Infection, Department of Biological Sciences, Imperial College, London, U.K.) (36) and detected by polyclonal donkey anti-rabbit Ig conjugated to CY3 (Jackson ImmunoResearch Laboratories) to identify adherent C. rodentium.

Colony counts
Fecal pellets, 0.25 ml of whole blood, distal colon (0.5 cm proximal to the anal canal), spleen, and liver were removed under sterile conditions, weighed, and homogenized. Homogenates were serially diluted and plated in triplicate to MAC. C. rodentium colonies were identified as pink, slightly translucent colonies, whereas colonizing E. coli were clearly distinguished from C. rodentium as lighter pink, nonmucoid, opaque colonies. Colonies were counted after 24 h of incubation at 37°C to determine the log10 CFU per gram of fecal material or tissue, or per milliliter of blood.
Ig ELISAs

Analyses were performed on fecal lysates or serum prepared from collected whole blood or PLs (LinBro, Flow Labor., ELS, MA; Lean, VA) were coated with heat-killed C. rodentium or E. coli or with a rabbit anti-mouse Ig capture Ab diluted 1/1000 in PBS (Zymed Laboratories). Because many capture reagents have poor specificity for the IgG-1 allele in C57BL/6 mice (37), capture of IgG2c Ig was conducted with a polyclonal goat anti-mouse IgG2c (1 mg/ml; Bethyl Laboratories, Montgomery, TX) diluted 1/1000 in PBS. Heat-killed C. rodentium or E. coli were prepared by suspending a centrifuged 1-h culture in PBS plus Sigma-Aldrich protease inhibitor mixture to an OD_{600} of 1.0. After incubation at 60°C for 1 h, culture aliquots were frozen at −80°C until use. Thawed aliquots were diluted 50× in PBS for coating of plates. Coated plates were washed in PBS plus 0.05% Tween 20, blocked for 1 h in soy blocking buffer, and incubated (Southern Biotechnology Associates, Birmingham, AL) conjugated to alkaline phosphatase, diluted 1/1000 in soy blocking buffer, and incubated (Southern Biotechnology Associates, Birmingham, AL) conjugated to alkaline phosphatase, diluted 1/1000 in soy blocking buffer, and incubated for 1 h at room temperature. After washing, plates were developed with p-nitrophenylphosphate (Sigma-Aldrich) and read in a Molecular Devices plate reader at OD_{405} of 1.0. After incubation at 60°C for 1 h, culture aliquots were frozen at −80°C until use. Thawed aliquots were diluted 50× in PBS for coating of plates. Coated plates were washed in PBS plus 0.05% Tween 20, blocked for 1 h in soy blocking buffer, and incubated (Southern Biotechnology Associates, Birmingham, AL) conjugated to alkaline phosphatase, diluted 1/1000 in soy blocking buffer, and incubated for 1 h at room temperature. After washing, plates were developed with p-nitrophenylphosphate (Sigma-Aldrich) and read in a Molecular Devices plate reader at OD_{405} of 1.0. Data were analyzed in SoftMax 4.0 and a determination of micrograms of total, isotype-specific Ig made against the standard curve for each plate. A minimum of five mice from at least two independent experiments were analyzed for each mouse strain. Ig measurements at 15 and 42 days postinoculation were obtained from unpaired mice.

Serum transfer studies

Five-week-old uninfected C57BL/6 mice served as donors of preimmune serum. Groups of 21-day-old donor C57BL/6 mice were inoculated with C. rodentium 6 wk (convalescent) or 2 wk (acutely infected) before inoculation of 21-day-old CD4-deficient recipients. Convalescent mice at 6 wk postinoculation were confirmed to be culture negative for C. rodentium from feces. At days 3, 4, and 5 postinoculation of CD4-deficient mice, whole blood was collected from donors and allowed to clot. Pooled serum was filter sterilized through 0.22-µm SpinX columns (Corning, Corning, NY) before immediate use. Groups of inoculated CD4-deficient recipients received 0.15 ml of 0.9% normal saline, or serum via tail vein injection. Aliquots of serum were saved for Ig ELISA to identify relative amounts of total and Citrobacter-specific Ab. Survival of CD4-deficient recipients was followed for 8 wk.

Ig purification and transfer

Serum was collected from preimmune, acutely infected, or convalescent wild-type mice as described and diluted 1/1 with high salt binding buffer (5.85 M NaCl, 1.5 M glycine (pH 9.0)), and then passed through a 0.45-µm filter before passage over Prosep protein A columns (Millipore, Bedford, MA). Columns were washed, and Ig was eluted with 10 ml of 0.1 M sodium citrate buffer at pH 5.5 followed by a second elution with 10 ml of 0.2 M glycine/HCl buffer at pH 2.5. Eluates were immediately collected into tubes containing 1 M Tris-HCl neutralization buffer. Collected fractions were pooled, dialyzed against 0.9% normal saline, concentrated against dry polyethylene-glycol 8000, and redialyzed against normal saline. Total, IgG, and E. coli-specific fractions were assayed by ELISA, and total protein content was determined by the Bradford Assay (Bio-Rad). Absence of other serum proteins was confirmed by performing serum protein electrophoresis and densitometry analysis on the purified material (SPIFE 3000; Helena Laboratories, Beaumont, TX) to observe presence of the μ and γ peaks, and absence of other serum proteins. The purification retained >90% of IgG fractions and 15–20% of the original serum IgM. Ig solutions were diluted to a concentration of 20 mg/ml in normal saline. A total of 2.0 mg of purified polyclonal Ig was administered i.v. to CD4-deficient mice on day 4 postinoculation with C. rodentium. Control mice received 0.2 ml of saline. Survival of infected recipients was followed for 8 wk.

Statistical analyses

Statistical analyses were performed in Prism 3.0 to allow calculation of means, SDs, and statistical significance by a nonparametric Kruskal-Wallis test. A value of p < 0.05 was considered significant.

Results

Mice lacking β7 integrins efficiently control infection with C. rodentium

Regional immune populations such as the gut-associated lymphoid tissue are assumed to play important functions in controlling infections localized to the intestine. We used a genetic approach to determine the importance of specific lymphocyte integrins on the outcome of infection with C. rodentium. Two integrins play key roles in lymphocyte homing to mucosal locations. The αβ2 integrin enables the attachment of T and B cells to mucosal addressin cell adhesion molecule-1 on high endothelial venules in Peyer’s patches and mesenteric lymph nodes, and on the capillary endothelium in lamina propria. Thus, the αβ2 integrin facilitates entry of B and T cells into the major gut mucosal compartment (38, 39). The αβ2 integrin mediates attachment of intraepithelial lymphocytes (IELs) to epithelial cells via its interaction with E-cadherin (40–42). αβ2 Integrin-deficient mice demonstrate significantly reduced numbers of IEL and lamina propria lymphocytes, whereas splenic and Peyer’s patch T cell numbers remain unaltered (26). β7 Integrin-deficient mice have a profound phenotype affecting both B cells and T cells. Only a few lymphocytes are detected in the lamina propria. Peyer’s patches, although present, are markedly reduced in size (28, 43). Despite profound reductions in the mucosal lymphocyte populations, wild-type, αβ2 integrin-deficient, and β7 integrin-deficient mice survived infection (Fig. 1B) with C. rodentium and cleared the organism 5–6 wk after inoculation (data not shown).

FIGURE 1. Survival among mouse strains lacking specific lymphocyte subsets. Mice were orally inoculated at 21 days of age with 5 × 10^8 (X) or 5 × 10^6 CFU (△) of C. rodentium.

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Mice lacking CD4+ T cells or TCR-αβ+ T cells succumb to infection with C. rodentium
To further investigate the role of specific T cell populations on the course of infection with C. rodentium, we undertook studies in CD4−/−, CD8α−/−, TCR-β−/−, TCR-δ−/−, and RAG2−/− mice. Deficiency of CD8+ T cells or TCR-γδ+ T cells, as with that of regional lymphocyte deficiencies mediated by β2 integrins, did not adversely impact survival of infection (Fig. 1, C and D). However, significant mortality was noted in mice lacking CD4+ T cells or TCR-αβ+ T cells (Fig. 1, E and F). CD4-deficient mice receiving 5 × 106 CFU of C. rodentium orally demonstrated a median survival of only 17 days and exhibited 100% mortality. Mice receiving 5 × 107 CFU/mouse demonstrated a median survival of 21 days, and only 33% of mice survived, although surviving mice successfully cleared C. rodentium from the feces by 6 wk postinoculation (data not shown). Similarly, TCR-β-deficient mice demonstrated mean survivals of 16 and 18 days for the two oral inocula, although no mice survived beyond 19 days for either inoculum (Fig. 1F). Animals lacking T and B cells (RAG2−/−) displayed an even more rapidly lethal course, with mean survival times of 12 and 10 days for oral inoculations with 5 × 105 and 5 × 106 CFU/mouse, respectively (Fig. 1G). These data demonstrate the necessity of CD4+ and TCR-αβ+ T cells in surviving the acute phase of infection with C. rodentium and show that CD8+ and TCR-γδ+ T cells play a negligible role in this process.

Mice lacking B cells succumb to infection with C. rodentium
Because RAG2-deficient mice, which lack mature T cells and B cells, had a more severe course of disease than TCR-β- and CD4-deficient mice, we sought to assess the role of B cells in survival of infection with C. rodentium. MuMT-deficient mice have a deletion of the Ig H chain that halts B cell development, although adult MuMT-deficient mice may possess IgA+ B cells in systemic and mucosal compartments (44). To control for the potential role of IgA in these mice, weaning-age MuMT C57BL/6 mice with undetectable levels of IgA in serum or feces before and during infection were studied. Strikingly, MuMT-deficient mice demonstrated markedly reduced mean survival times of 10 days at the two oral doses (Fig. 1H), implicating a necessary role for B cells in surviving acute infection with C. rodentium.

C. rodentium infection produces disruptions in epithelial integrity secondary to abscess formation in both wild-type and immunodeficient mice
Wild-type weaning-age mice inoculated with C. rodentium had detectable attaching and effacing lesions in the distal colon 3 days postinoculation (data not shown). Extended areas of luminal colonization were present by 7 days as noted by intimately adherent cocccobacillary forms on H&E and Steiner silver stain (Fig. 2B). By 15 days, animals demonstrated epithelial hyperplasia and the presence of CD4+ T cells situated around the proliferative crypts of Lieberkühn in the distal colon (data not shown). Steiner silver stains demonstrated the presence of C. rodentium adherent to the luminal face of the colon or upper third of colonic crypts of Lieberkühn (Fig. 2F). Colonization of the organism along the entire colonic crypt-cuff axis occurred in <1% in 1000 colonic crypts. Although the colonic epithelium remained grossly intact, breaks in epithelial integrity were apparent over rare lamina propria abscesses that formed beneath areas densely colonized with C. rodentium (Fig. 2G). Cocccobacilli, rods, and spirochetes could be visualized in the lamina propria of these areas, indicating a sufficient breach of the epithelial barrier to permit entry of pathogenic and commensal species (Fig. 2K). Similar histopathology was found in CD8α- and TCR-δ-deficient mice (data not shown).

We next examined the histopathological findings in mice that succumbed to infection. CD4-deficient mice demonstrated breaks in epithelial integrity associated with areas of abscess formation comparable with that of wild-type mice (Fig. 2H). TCR-β-deficient mice demonstrated more frequent focal abscesses in the anorectal mucosa than CD4-deficient mice (data not shown). Although mice lacking CD4+, or TCR-β+ T cells developed mild to moderate colonic immunopathology during acute infection, these mice consistently developed focal abscesses in liver and spleen at 15 days postinoculation, suggesting systemic spread of infection (Fig. 2, compare M with N).

RAG2- and MuMT-deficient mice displayed more severe colonic damage and indications of systemic infection as early as 7 days postinoculation. These mice demonstrated dense colonization of the distal colon with areas of abscess formation in the lamina propria, resulting in distinct breaches across the epithelial barrier (Fig. 2, D and E). Mice surviving to 15 days demonstrated extensive colonization of colonic crypts of Lieberkühn with C. rodentium, significant abscess formation in the lamina propria, transmural inflammation, and serositis (Fig. 2I, asterisk). Loss of goblet cells was noted in inflamed areas heavily colonized with C. rodentium. Multiple breaches across the epithelial layer were noted. RAG2-deficient mice showed extreme destruction of the anorectal mucosa with prominent neutrophilic infiltrates and necrosis of the overlying epithelium (Fig. 2E). In both MuMT- and RAG2-deficient mice, dense microcolonies of bacteria filled areas once representing the lumens of colonic crypts, and congested lymphatics of the lamina propria (Fig. 2, I and L). Both C. rodentium and components of the normal flora resided in necrotic areas, in direct contact with underlying tissues (Fig. 2L). Furthermore, isolated patches of attaching and effacing lesions caused by adherent C. rodentium were present in jejunum and ileum of some B cell-deficient mice (data not shown). As with all mice that succumbed to infection, both MuMT- and RAG2-deficient mice demonstrated numerous focal abscesses in the liver during acute infection (data not shown).

Mice lacking B cells, CD4+, or TCR-αβ+ T cells develop polymicrobial infections in internal organs during acute infection with C. rodentium
To determine whether immunopathology noted in colonic and systemic locations correlated with the bacterial burden in these tissues, we measured the burden of C. rodentium in the distal colon, liver, spleen, and blood of infected mice. Mice in our facility are colonized with a nonpathogenic strain of E. coli. This strain, in addition to commensal anaerobes, were frequently cultured during CFU analyses of internal organs (data not shown). Thus, we used the ability to culture commensal E. coli as an indicator of the polymicrobial nature of the sepsis that developed in immunodeficient mice.

As early as 3 days postinoculation, MuMT−/− and RAG2−/− mice demonstrated 3 logs greater CFU of C. rodentium per gram of tissue in the distal colon, as compared with other mouse strains (data not shown). By 7 days postinoculation, wild-type, TCR-δ-deficient, and CD8α-deficient mice demonstrated no significant difference among their mean log10 of C. rodentium per gram of colonic tissue (means of 7.05, 6.48, and 6.60, respectively; Fig. 3A). Although TCR-β- and CD4-deficient mice demonstrated higher mean colonic burdens of C. rodentium (8.16 and 8.21, respectively), as compared with the first group of mice, these burdens were not significantly different from wild-type animals (p = 0.10). However, at this early stage of acute infection, MuMT- and RAG2-deficient mice demonstrated mean burdens of C. rodentium...
C. rodentium mice, the CFU loads of liver, and blood, and 50% of mice had detectable E. coli. MuMT-decient mice demonstrated CFU of E. coli to a noninvasive C. rodentium (arrowhead).

By 15 days of infection, the colonic burden of C. rodentium in wild-type mice did not differ significantly from mice with T cell defects (Fig. 3B). Although the gap between colonic burden in these mice and MuMT- and RAG2-deficient mice diminished, both MuMT- and RAG2-deficient mice maintained half-log higher CFU burdens of 10.06 and 10.05, respectively, as compared with wild-type mice (p < 0.0023).

Despite the negligible differences in colonic burdens of C. rodentium among wild-type, CD4-deficient, and TCR-β-deficient mice, the CFU loads of C. rodentium and of commensal E. coli cultured from blood, liver, and spleen revealed profound differences during acute infection. No CFU were cultured from internal organs of any mouse strain at 3 days postinoculation (data not shown). However, concomitant with the development of breaches in the colonic epithelium by 7 days postinoculation, all infected RAG2- and MuMT-deficient mice demonstrated C. rodentium in the spleen, liver, and blood, and 50% of mice had detectable E. coli in blood, indicating the polymicrobial nature of the sepsis (Fig. 3, C and E, and data not shown). No commensal bacteria were cultured from internal organs from wild-type mice or mice with T cell deficiencies (Fig. 3C). However, by 15 days, CD4- and TCR-β-deficient animals demonstrated a significant burden of C. rodentium and E. coli cultivable from blood, liver, and spleen (Fig. 3, D and F, and data not shown), whereas <10% of infected wild-type, TCR-β-deficient, or CD8α-deficient mice demonstrated CFU of C. rodentium in remote tissues, and no mice demonstrated cultivable E. coli from spleen (Fig. 3C). Thus, evidence of systemic infection, indicated by abscess formation in liver and spleen secondary to a polymicrobial sepsis occurring across damaged, infected mucosa, correlated with failure to control the primary mucosal infection caused by C. rodentium and contributed to the death of infected mice.

**CD4- and TCR-β-deficient mice lack an early IgM response to C. rodentium**

We hypothesized that early circulating Ig generated against C. rodentium might be critical to survive acute infection. If so, the susceptibility of CD4- and TCR-β-deficient mice to a noninvasive

![Image of histological sections](https://example.com/image.png)
mucosal pathogen might result from a defect in mounting an initial T cell-dependent Ig response against entering organisms. To test this hypothesis, we measured total and Citrobacter-specific Ab among infected mouse strains. We found that total serum IgM levels were similar among wild-type and TCR-β-, TCR-δ-, CD4- and CD8α-deficient mice at 15 days of infection (mean values of 2621 ± 514, 2278 ± 437, 2186 ± 253, 2538 ± 598, and 2292 ± 488 μg/ml IgM, respectively). However, CD4- and TCR-β-deficient mice possessed 6- and 20-fold lower Citrobacter-specific IgM, respectively, as compared with wild-type mice (p < 0.0077; Fig. 4A). Citrobacter-specific serum IgG levels at this early stage of the infection were also significantly lower in TCR-β- and CD4-deficient mice (p < 0.0022; Fig. 4B). Citrobacter-specific serum and fecal IgA were not detectable above background among all mouse strains at 15 days (data not shown). Thus, death from infection during the first 2–3 wk after challenge with C. rodentium correlated with failure to mount a strong pathogen-specific IgG response.

We also examined Ab responses in surviving mice at later time points (6 wk) after challenge. By 6 wk postinoculation, wild-type mice developed a strong anti-Citrobacter serum IgG response characterized by a predominance of pathogen-specific IgG2b, IgG2c, and IgG3 isotypes (Fig. 4, E–G). Serum IgM levels at 6 wk in CD4-deficient mice surviving the lower inoculum of 5 × 10^7 CFU approximately doubled those of CD4^-/- mice at 15 days (Fig. 4C), and pathogen-specific IgG1 levels were equivalent to those of wild-type mice (D). However, CD4-deficient animals demonstrated >10-fold lower levels of pathogen-specific IgG2b, IgG2c, and IgG3 isotypes (Fig. 4, E–G), comparable with total reductions in circulating levels of these IgG classes as compared with wild-type mice. Although circulating, pathogen-specific serum IgA was negligible in wild-type and CD4-deficient mice (Fig. 4H), both groups demonstrated comparable levels of total and anti-Citrobacter IgA in feces (Fig. 4F).

Transfer of serum from actively infected or convalescent wild-type mice protects CD4-deficient mice from succumbing to infection with C. rodentium

To determine whether factors in serum could circumvent the deficiency of CD4^+ T cells, CD4-deficient mice inoculated with the normally lethal inoculum of 5 × 10^8 C. rodentium received i.v. injections of saline or serum from either preimmune, acutely infected, or convalescent wild-type mice on days 3, 4, and 5 postinfection. Transfer of serum from actively infected or convalescent mice protected all recipients (Fig. 5). Transfer of wild-type, preimmune sera protected 17% of CD4-deficient recipients, whereas all animals receiving saline succumbed to infection (Fig. 5).

Serum IgM and IgG fractions from acutely infected or convalescent mice protect CD4-deficient mice from succumbing to infection

To verify that serum Ab conferred protection in mice lacking CD4^+ Th functions, we purified polyclonal serum IgG and IgM fractions from preimmune, acutely infected and convalescent C57BL/6 mice (Table I). Intravenous administration of 2.0 mg of purified Ab from convalescent wild-type mice protected all CD4-deficient recipients, whereas transfer of purified Ab from acutely infected mice protected 80% of CD4-deficient recipients (Fig. 6). Transfer of saline or 2.0 mg of preimmune Ab had no effect. Furthermore, by 6–8 wk postinoculation, surviving CD4-deficient recipients successfully cleared C. rodentium from the colon and developed a pathogen-specific secretory IgA response comparable with that of wild-type mice (data not shown). These data verify that the acute and convalescent serum Ab responses generated in wild-type mice can circumvent deficiency of CD4^+ T cells, allowing CD4-deficient recipients to survive acute infection and eventually resolve disease.

Discussion

Luminal infection with the attaching and effacing pathogen C. rodentium produces breaks in the colonic epithelium, particularly over heavily colonized surfaces. These areas create passive portals in both wild-type and immunodeficient mice through which C. rodentium and commensal species may gain entry to deeper tissues. The escape of small numbers of pathogenic and commensal bacteria from active sites of mucosal infection has the potential to create sites of polymicrobial infection in nonmucosal locations, and results in significant damage to end organs in Ig-deficient mice, or in animals lacking Th cell functions. Although human EPEC infections elicit neutrophilic infiltrates in the small bowel, as opposed to colonic CD4^+ T cell infiltrates elicited in C. rodentium-infected mice, both infections cause disruptions in the epithelial barrier, a process that creates the potential for polymicrobial sepsis and local or disseminated abscess formation with pathogenic and commensal species. Given the severity of EPEC infection in HIV^+ humans or SIV^+ rhesus macaques (2, 3), we hypothesize that the early T cell-dependent serum Ab may also play a crucial role in EPEC infection.

The need for B cells, as exemplified by the lethality in acutely infected MuMT-deficient mice, further highlights the protective role of the early pathogen-specific Ig response during infection with attaching and effacing pathogens, and is consistent with similar findings reported in RAG1-deficient mice (45). However, the
The complete absence of appreciable Ig, or other B cell products, in mucosal and systemic compartments in MuMT- and RAG2-deficient mice affects the course of disease in both the colonization and acute phases of infection, as well as the resulting tissue pathology in both locations. B cell-deficient mice consistently developed higher colonic CFU of *C. rodentium* earlier in infection than mice with T cell deficits, indicating poorer luminal control of initial events in colonization and proliferation of pathogenic bacteria. The degree to which secretory IgA or other B cell products contribute to the initial control and resolution of the mucosal infection remains to be further evaluated.

Although CD4-deficient mice demonstrated equivalent degrees of colonic pathology and similar colonic burdens of *C. rodentium* as wild-type mice, these animals exhibited significant sepsis, development of abscesses in end organs, and mortality. CD4+ T cells...
may play a variety of roles during infection with attaching and effacing pathogens, including interacting with epithelial and phagocytic cells to limit passive or active entry of pathogenic and commensal species, and to assist with the development of mucosal Ab responses. Importantly, our data emphasize that, during acute infection with a mucosal pathogen, Th cells play a crucial role in the generation of early pathogen-specific serum Ab.

Intravenous administration of polyclonal serum IgG and IgM, before the onset of symptomatic disease, successfully circumvented the deficiency of Th cells in CD4-deficient mice. The need for early T cell-dependent Ab complement studies in mice deficient for IFN-γ or for IL-12 that also demonstrated significant lethality from infection with C. rodentium (46). Although these cytokines affect aspects of immunity to C. rodentium, an understanding of their impact on the development of a protective Ab response and their effects on innate and other adaptive immune functions, will provide a better understanding of the host responses needed to survive and clear mucosal infections.

Transfer of Ig fractions containing pathogen-specific IgM from acutely infected mice suggest that this isotype plays a critical role during acute infection. However, we cannot rule out the possibility that lesser concentrations of reactive IgG produced during acute infection may be important for functions including fixation of complement and clearance of bacteria by phagocytic cells that contribute to the host’s ability to survive acute infection. Conversely, the ability of adoptively transferred CD4-deficient mice to mount effective secretory IgA responses, and clear C. rodentium from the colon, suggests that mechanisms independent of CD4+ Th cells may be adequate to resolve infection.

Given that C. rodentium has been considered a self-limited, colonizing infection limited to the lumen of the colon, we expected that the gut-associated lymphoid tissue would be the critical site of immune response to control infection. However, the survival and clearance of infection in αc and in β2 integrin-deficient mice suggest that lymphoid populations established in Peyer’s patches, lamina propria, IEL, and other gut locations in wild-type mice were dispensable for survival and clearance of infection. Furthermore, entry of lymphocytes into mucosal compartments via the αcβ2 integrin-mucosal addressin cell adhesion molecule-1 association is not a requirement for mice to successfully survive and resolve infection. Besides providing understanding concerning the pathogenesis of mucosal infections in the gut, these studies provide important insights that may assist in the development of vaccines against attaching and effacing pathogens, including use of systemic vaccination strategies, to protect susceptible populations. Our findings also underscore the need to treat immunocompromised individuals acquiring these infections with antibiotic regimens that will treat both the mucosal as well as potential systemic infection.

FIGURE 6. Adoptive transfer of serum IgGs to CD4−/− mice. Survival in CD4-deficient mice receiving 2.0 mg of polyclonal serum IgG and IgM from preimmune (n = 6 mice; □), acutely infected (n = 9 mice; ■), convalescent mice (n = 9 mice; ○), or 0.2 ml of saline (n = 6 mice; ◆) at 4 days postinoculation with 5 × 10⁵ C. rodentium.

Table 1. Percentages of total, C. rodentium- and E. coli-specific IgG and IgM in purified serum Ab preparations transferred to CD4−/− recipient mice

<table>
<thead>
<tr>
<th></th>
<th>Total IgM (%)</th>
<th>Total IgG (%)</th>
<th>Anti-CR IgM (%)</th>
<th>Anti-CR IgG (%)</th>
<th>Anti-EC IgM (%)</th>
<th>Anti-EC IgG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preimmune</td>
<td>1.24</td>
<td>98.76</td>
<td>0.12</td>
<td>0.10</td>
<td>0.21</td>
<td>0.39</td>
</tr>
<tr>
<td>Acute</td>
<td>12.91</td>
<td>87.09</td>
<td>3.99</td>
<td>0.89</td>
<td>1.64</td>
<td>1.27</td>
</tr>
<tr>
<td>Convalescent</td>
<td>4.41</td>
<td>95.58</td>
<td>0.95</td>
<td>11.82</td>
<td>0.52</td>
<td>2.38</td>
</tr>
</tbody>
</table>

*All percentages were calculated relative to total Ab (IgG plus IgM) in solution (20 mg/ml). Values represent averages from individual purifications used to obtain serum IgG and IgM for injection into recipient CD4−/− mice. Anti-CR, Anti-C. rodentium-specific Ab; anti-EC, anti-E. coli-specific Ab.

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References


