Mycobacterium avium paratuberculosis Invades Human Small-Intestine Goblet Cells and Elicits Inflammation

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Crohn disease is a chronic inflammatory bowel disease of unknown etiology. Mycobacterium avium paratuberculosis (MAP) was found in the gut of patients with Crohn disease, but causality was not established. Fully developed, germ-free human small intestine and colon were established by subcutaneous transplantation of fetal gut into SCID (severe combined immunodeficiency) mice thereafter infected by direct intraluminal inoculation of MAP. We have found that MAP actively invades the human gut epithelial goblet cells of the small intestine, inducing severe tissue damage and inflammation. These observations indicate that MAP can specifically colonize the normal human small intestine and can elicit inflammation and severe mucosal damage.

Crohn disease (CD), a chronic debilitating inflammatory bowel disease with a complex etiology and an increasing incidence worldwide [1], is caused by a dysregulated immune response to one or more unknown antigens, most likely bacterial in origin, in genetically susceptible hosts. It is widely accepted that environmental factors, such as exposure to certain microorganisms, might trigger the disease in individuals carrying one or more susceptibility genes. Among the suggested organisms are commensal enteric bacteria, psychrotrophic bacteria (e.g., Listeria and Yersinia organisms) that survive in refrigerated food (the cold-chain hypothesis), and various microbial components, such as flagellins and nucleic acids [2]. However, undoubtedly the most enduring infectious hypothesis regarding CD is its association with Mycobacterium avium paratuberculosis (MAP) [3]. MAP is the etiologic agent of a severe gastroenteritis in ruminants that is known as “Johne disease” [4]. For decades, CD’s resemblance to Johne disease led researchers to point to the possibility that the causative organism of Johne disease is also associated with CD. Indeed, MAP was detected in breast milk, blood, and intestinal tissue of patients with CD but was frequently found also in healthy individuals [3]. Use of Koch’s postulates to investigate the etiologic role that MAP plays in CD cannot be fully justified without infection studies of the human gut of genetically susceptible individuals [5]. Therefore, the role that MAP plays in CD remained controversial.

In Johne disease, MAP first colonizes the intestine during infancy, remains asymptomatic for several years, and causes disease only in subpopulations of infected animals. We therefore hypothesize that, if MAP is involved in CD, it might initially colonize the human gut at an early age and trigger CD only later, should the colonized individual be genetically susceptible. We therefore tested the capacity of MAP to establish this early colonization in human intestine and to react specifically with the intestinal epithelia and the immune system. We have used human fetal intestinal xenografts in the severe combined immunodeficiency (SCID) mouse model (SCID-HU-INT) [6] to study the interaction between MAP and the human gut and the mouse innate immune system. Human intestinal xenografts implanted into the subcapsular region of a SCID mouse become vascularized with murine endothelial cells and develop a lumen, with a morphologically precise mucosal layer containing villi and crypts, lamina propria, and muscularis layers [6]. Intestinal xenografts are even capable of nutrient absorption and peristalsis [7]. This model, which closely mimics natural human infection, has now been used to study the interactions between several enteric pathogens and the human intestine. Intestinal diseases studied by use of the SCID-HU-INT model include bacillary (Salmonella and Shigella), amoebic, and Cryptosporidium dysentery [8]. Using this model system, we show in this report that MAP can specifically colonize the normal human small intestine via goblet-cell invasion and that it elicits inflammation and severe mucosal damage.

**Materials and methods.** MAP strain K-10 was grown at 37°C in Middlebrook’s 7H9 (Difco) supplemented with 0.2% glycerol, 10% OADC, Mycobactin J (Allied Monitor) at 2 mg/L, 0.05% Tween 80, and 1% cyclohexamide (M-OADC-TW...
infused MAP into 43 intestinal xenografts, nonpathogenic MS crine cells (data not shown). Using intraluminal injection, weations of enterocytes, goblet cells, Paneth cells, and enteroendo-

Appendix, which is available only in the electronic version of the Journal. In the Appendix, which is available only in the electronic version of the Journal, we observed that numerous MAP bacteria adhered to the mucus layer on the apical side of the intestinal villous epithelium (figure A2A and B in the Appendix, which is available only in the electronic version of the Journal). Although most enterocytes were not invaded, specific invasion of goblet cells in the villi and crypts of the small intestine was observed (figure 1B; also see figure A2 in the Appendix, which is available only in the electronic version of the Journal).

Interestingly, different results were obtained when xenografts taken from different donors were infected; however, the results of infection of different xenografts taken from the same donor were similar. These results indicate that some individuals are more susceptible to initial MAP infection. A second interesting observation is that, although the model that we used combined human gut with the murine immune system, the human gut infection elicited a murine innate response, as described below.

In 3 xenograft donors, epithelial invasion by MAP was associated with severe changes characterized by patchy distribution of multifocal disseminated areas of epithelial damage, hemorrhages, and inflammation (figure 1C; also see figure A3A and A4 in the Appendix, which is available only in the electronic version of the Journal). Inflammation was characterized by recruitment of round and polymorphonuclear cells, perivascular cuffing in the submucosa (figure A4 in the Appendix, which is available only in the electronic version of the Journal), and diffuse infiltration of CD68-positive macrophages and polymorphonuclear cells into the lamina propria of the crypts and villi (figure 1D and E). In addition, inflammatory changes were associated with elevated concentrations of IL-1β, TNF-α, and IL-6 in tissue homogenates, compared with PBS-challenged donor-matched control xenografts (figure 2). Importantly, MAP invasion of goblet cells, the ensuing tissue damage, and the inflammation were specific to the small intestine, whereas the reciprocal colon xenografts infected by MAP were not affected (figure A5 in the Appendix, which is available only in the electronic version of the Journal). In the other 6 xenograft donors, MAP still invaded the goblet cells but the infection was associated with milder inflammation and no epithelial damage.

In all cases of PBS-challenged control xenografts, only small numbers of CD68-positive mouse macrophages invaded the lamina propria of the crypts and villi of the germ-free intestinal xenografts (figure A3B and C in the Appendix, which is available only in the electronic version of the Journal). As an additional control, donor-matched xenografts were infected by the nonpathogenic MS. Numerous organisms were observed to adhere to the mucus layer on the apical side of the intestinal villous epithelium; however, goblet cells were only sparsely invaded (figure A6A and B in the Appendix, which is available only in the electronic version of the Journal). Epithelial damage, inflammation, and CD68-positive macrophage infiltration were either ab-

BRIEF REPORT • JID 2009:199 (1 February) • 351
sent or extremely mild (figure A6C–F in the Appendix, which is available only in the electronic version of the Journal), and tissue concentrations of IL-1β, TNF-α, and IL-6 were lower (figure 2).

**Discussion.** Although considerable evidence exists to support the specific association between MAP and CD, causality remains unproven. Opponents of the “MAP theory” frequently
argue that the organism simply acts as a secondary invader in a host with a compromised intestinal barrier. Therefore, causality can only be established by "infection studies in children" [5]; however, because such research is totally unacceptable and unethical, the only current alternative is the "humanized mouse" model. The results of the present study, which used the SCID-HU-INT model, indicate that MAP has the capacity for initial colonization of the human small intestine and that some individuals are more sensitive to MAP infection. Interestingly, the colonization and inflammatory response were specific to the small intestine, which is similar to what has been found in Johne disease in ruminants, in which infection and chronic granulomatous enteritis are commonly limited to the ileum [4]. Furthermore, we have found that MAP infecting the human small intestine exhibits dramatic tropism toward goblet cells, which is associated with acute inflammatory response and epithelial damage. The bacterium seems to adhere to the mucus layer of the human gut epithelium and to migrate, via an unknown mechanism, to the apical surface of the goblet cells, where the cells are invaded and colonized. Schlieg et al. [10] have described MAP invasion of ileum goblet cells in bovine organ cultures, although it should be noted that this had not been observed during the preceding century of research on Johne disease in ruminants, in which infection and chronic granulomatous enteritis are commonly limited to the ileum [4]. Furthermore, we have found that MAP infecting the human small intestine exhibits dramatic tropism toward goblet cells, which is associated with acute inflammatory response and epithelial damage. The bacterium seems to adhere to the mucus layer of the human gut epithelium and to migrate, via an unknown mechanism, to the apical surface of the goblet cells, where the cells are invaded and colonized. Schlieg et al. [10] have described MAP invasion of ileum goblet cells in bovine organ cultures, although it should be noted that this had not been observed during the preceding century of research on Johne disease in ruminants, in which infection and chronic granulomatous enteritis are commonly limited to the ileum [4]. Furthermore, we have found that MAP infecting the human small intestine exhibits dramatic tropism toward goblet cells, which is associated with acute inflammatory response and epithelial damage. The bacterium seems to adhere to the mucus layer of the human gut epithelium and to migrate, via an unknown mechanism, to the apical surface of the goblet cells, where the cells are invaded and colonized. Schlieg et al. [10] have described MAP invasion of ileum goblet cells in bovine organ cultures, although it should be noted that this had not been observed during the preceding century of research on Johne disease in ruminants, in which infection and chronic granulomatous enteritis are commonly limited to the ileum [4].

Figure 2. Concentrations (logarithmic scale) of mouse inflammatory cytokines interleukin (IL–1β), tumor-necrosis factor (TNF–α), and IL–6 in homogenized human intestinal xenografts after intraluminal challenge with 5.0 × 10^7 of Mycobacterium avium paratuberculosis (MAP) or, as negative controls, with either PBS or 5.0 × 10^7 of M. smegmatis (MS); horizontal bars indicate mean concentrations. Data represent results for 8 donor fetal xenografts; some data points are superimposed. *P < .05; **P < .01.

Although we have observed acute infection, inflammation, and tissue damage in the human small intestine, none of these were observed after similar challenge of bovine fetal intestinal xenografts in SCID mice (SCID-BO-INT) (N.Y.S., unpublished results). These results are in agreement with numerous experimental infection studies of various ruminants species in which inflammation or gut pathology was not observed during the early stages after challenge [14]. There seem to be important differences between MAP’s early host-pathogen interactions in the gut of some humans and its early host-pathogen interactions in the natural ruminant host.

The animal model used in the present study includes a combination of murine and human innate immune constituents while B or T lymphocytes of either species are absent. Murine blood monocytes migrate into the lamina propria of the human intestinal xenograft and are able to elicit an inflammatory response to microbial organisms and their products in the lumen. Paneth cells were clearly visible in the crypts of the epithelium, and resistin-like molecule β was highly expressed in human small-intestinal xenografts, whereas human interleukin was highly expressed in colon xenografts but not in small-intestinal xenografts (A.L.-K. and N.Y.S., unpublished results). Although a dysregulated adaptive immune response to microbial constituents in the gut is considered to be the underlying cause of CD, the innate response might be the triggering element [1]. The results of the present study show that MAP bacteria can infect and trigger the innate immune system to elicit inflammation in the im-
mature and germ-free human small intestine. Fetal infection is known to occur in chronically infected pregnant ruminants, such as cows, sheep, and goats. Years later, many of these fetal infections can lead, via an unknown mechanism, to flare-ups of clinical Johne disease. MAP bacteria have been identified in the blood of patients with CD and even in the milk of breast-feeding women. Fetal or neonatal infection by MAP bacteria in genetically susceptible individuals might elicit an immune response deranging the gut immune system’s future tolerance of normal microbial constituents in the gut. Therefore, CD or a subset of it might be a delayed complication of fetal or neonatal exposure of the gut to MAP, rather than a bone fide infectious disease caused by MAP. The considerable differences between fetal donors further underscore the critical importance of the genetic make-up of the host in this disease. Genetic analysis of fetal donors to identify known CD-susceptibility genes, such as NOD2, ATG16L, and IL23R [1, 15], might further substantiate our results and are under way.

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References

Figure A1. Human fetal intestinal xenograft in SCID (severe combined immunodeficiency) mice. A, Fetal gut immediately after being transplanted subcutaneously (also see figure 1A). Also shown are representative microscopic images of hematoxylin-eosin–stained formalin-fixed paraffin sections (B, D, and E) and fluorescence-stained cryosections (C) with anti-cytokeratin antibodies (green) and 4′,6-diamidino-2-phenylindole (blue), from fully differentiated small-intestinal (B and C) and colon (D and E) xenografts. (Scale bars, 500 μm [B and D] and 50 μm [C and E])
Figure A2. Results of Mycobacterium avium paratuberculosis (MAP) invasion of human intestinal epithelial goblet cells, as shown in representative microscopic images (original magnification, ×100) of tissue sections of human small-intestinal xenografts in SCID (severe combined immunodeficiency) mice 3 days after intraluminal challenge with 5.0 × 10⁷ of MAP bacteria. Xenograft cryosections were stained with anti-MAP antibody either alone (green channels in panel A) or combined with 4′6-diamidino-2-phenylindole (blue channels in panel B) and phalloidin-rhodamine (red channels in panel B). The red and green channels observable in figure 1B are also shown in panels C and D, respectively, of this figure. Epithelial goblet cells were only faintly stained by phalloidin-rhodamine (white arrow in panel C). MAP bacteria adherent to the brush border also are indicated (yellow arrows in panels A and B), as are those that are adherent to mucus excreted by goblet cells and that specifically invade the goblet cells of the villous epithelium (white arrows in panels B and D).

Figure A3. Enteritis induced by Mycobacterium avium paratuberculosis infection of human small intestine, as shown in representative microscopic images of tissue sections of human small-intestinal xenografts in SCID (severe combined immunodeficiency) mice 3 days after intraluminal challenge either with 5.0 × 10⁷ of MAP bacteria (A) or with PBS (B and C). Hematoxylin-eosin–stained formalin-fixed sections (A), as well as cryosections (B and C) stained with anti-CD68 (green) combined with 4′6-diamidino-2-phenylindole (blue) and phalloidin-rhodamine (red), are shown. Infection is associated with the focal area of epithelial damage, hemorrhage, and inflammation, characterized by the presence of murine macrophages and neutrophil infiltration (A). This is better seen in figure 1C, which is an enlargement of the boxed area in panel A of this figure, and in figure A4C and D. Similar pathology was not seen in either the PBS-challenged control xenografts (B and C) or the M. smegmatis (MS)–challenged control xenografts (see figure A6C–F). MAP infection elicits massive recruitment of murine CD68-positive macrophages into the lamina propria of the submucosa, crypts, and villi (figure 1D and E); however, both in the PBS-challenged control xenografts (B and C) and in the MS-challenged control xenografts (figure A6E and F), these areas are only sparsely populated by CD68-positive murine macrophages. (Scale bars, 500 µm [A], 200 µm [B], and 50 µm [C]).
Figure A4. Severe enteritis induced by *Mycobacterium avium paratuberculosis* infection of the human small intestine, as seen in representative microscopic images of hematoxylin-eosin–stained formalin-fixed tissue sections of human small-intestinal xenografts in SCID (severe combined immunodeficiency) mice 3 days after intraluminal challenge with $5.0 \times 10^7$ of MAP bacteria. Focal areas of epithelial damage, hemorrhage, and inflammation are characterized by the presence of murine macrophages and neutrophil infiltration (A and B, the latter of which is an enlargement of the boxed area in panel A). The sharp transition between normal villi and the focal area of epithelial damage and inflammation is clearly seen in panels C and D. Focal areas of inflammation (E), perivascular cuffing in the lamina propria of the crypts (F), and neutrophil infiltration of damaged crypts (G) are also shown. (Scale bars, 500 μm [A], 200 μm [B], and 50 μm [C–G])
Figure A5. Tissue sections of human colon xenografts in SCID (severe combined immunodeficiency) mice 3 days after intraluminal challenge with $5.0 \times 10^7$ of *Mycobacterium avium paratuberculosis* (MAP) bacteria. MAP bacteria do not invade the human colon goblet-cell epithelium and only sparsely adhere to the villous mucus layer. Representative microscopic images of hematoxylin-eosin–stained formalin-fixed tissues (A and B) and of fluorescence-stained cryosections combined with phase-contrast images (C and D) are shown. Normal epithelial structures are maintained after infection by MAP bacteria, without signs of inflammation or abnormal leukocyte infiltration (A and B). Nuclei and bacterial DNA are stained with Sytox orange (red in panels C and D), and clumps of MAP bacteria visible in the lumen (white arrow in panel C) only sparsely adhere to the epithelial mucus (white arrowhead in panel C). (Scale bars, 500 μm [A], 50 μm [B], and 20 μm [C and D]).
Figure A6. *Mycobacterium smegmatis* (MS) infection of the human small intestine. MS infection is not associated with epithelial destruction and inflammation. Representative microscopic images of fluorescence-stained (A, B, E, and F) and hematoxylin-eosin–stained formalin-fixed (C and D) tissue sections of human small-intestinal xenografts in SCID (severe combined immunodeficiency) mice 3 days after intraluminal challenge with $5.0 \times 10^7$ of MS bacteria are shown. Nuclei and bacterial DNA are stained by Sytox orange (black and white image in panel A), and clumps of MS bacteria visible in the lumen (yellow arrow in panel A) only sparsely adhere to the epithelial mucus (white arrows in panel A). Xenografts stained with immunofluorescence for MS bacteria (green areas in panel B) combined with 4′,6-diamidino-2-phenylindole (blue areas in panel B) and phallolidin-rhodamine (red areas in panel B) also are shown. Epithelial goblet cells are not stained by Sytox or phallolidin-rhodamine and appear as black holes (arrowheads in panels A and B). MS bacteria adherent to the brush border (colocalized with phallolidin-rhodamine) also are shown (white arrows in panel B). Goblet cells are only sparsely invaded by MS bacteria (arrowheads in panels A and B). Normal epithelial structures and the lamina propria of the crypts and villi are only sparsely populated by murine round cells and neutrophils (C and D), as is also demonstrated by cryosections (E and F) stained with immunofluorescence for CD68 (green) combined with 4′,6-diamidino-2-phenylindole (blue) and phallolidin-rhodamine (red). (Scale bars, 20 μm [A and B], 200 μm [C and E], and 50 μm [D and F]).