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Johne's Disease, Inflammatory Bowel Disease, and *Mycobacterium paratuberculosis*

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Abstract

Johne's disease is a chronic diarrhea affecting all ruminants. *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a slowly growing mycobacteria, is the etiologic agent. There is also a concern that MAP might be a causative agent of some cases of inflammatory bowel disease in humans, especially Crohn's disease. Food products including pasteurized bovine milk have been suggested as potential sources of human infection. This review addresses microbial factors that may contribute to its pathogenicity. In addition, the experimental evidence defining MAP as the cause of Johne's disease and the issues and controversies surrounding its potential pathogenic role in humans are discussed.

Contents

Introduction

Mycobacteria (taxonomically referred to as the genus *Mycobacterium*) include a group of high GC gram-positive microorganisms comprising more than 100 species. Although most of these species are saprophytic, important human and animal pathogens have been identified. Pathogenic members are usually characterized by their slow growth in culture, with generation times of 12 to 24 h, whereas nonpathogenic members grow considerably faster. Taxonomical classifications place the genus *Mycobacterium* as a relative of the genera *Corynebacterium*, *Nocardia*, *Rhodococcus*, and *Streptomyces*. Mycobacteria are characterized by their acid-fastness and lipid-rich cell wall. The main pathogenic species include *Mycobacterium avium*, *M. bovis*, *M. leprae*, and *M. tuberculosis*. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the etiologic agent of Johne's diseases in ruminants and is also associated with Crohn's disease in humans (72).

The Microorganism

M. avium subsp. *avium* (MAV) and *M. intracellulare* have long since been recognized as opportunistic human pathogens for patients with chronic respiratory infections, such as obstructive lung disease. More recently, these microorganisms have been associated as major opportunistic pathogens of immunosuppressed individuals, especially AIDS patients (88). Because of this close clinical relationship in human medicine, both species are often called the *M. avium* complex (MAC). Technically, this term should apply to all the subspecies of *M. avium*, including MAP and *M. avium* subsp. *silvaticum*. However, this is rarely recognized in human infectious disease. Only recently have a limited number of MAP infections in AIDS patients been reported (134), and no cases have implicated *M. avium* subsp. *silvaticum*. A key report regarding the close relationship among the subspecies of *M. avium* was the study by Thorel et al. (160). Several isolates were subjected to a battery of genotypic and phenotypic tests and correlated with prior studies on DNA-DNA hybridization tests. This analysis led to the subdivision of the *M. avium* group into the three subspecies mentioned above. MAP is the subspecies with the slowest growth rate, with a generation time of over 20 h that varies with

the number of microorganisms present in the inoculum (99). Moreover, MAP is the only subspecies that depends on the siderophore mycobactin for growth, especially when grown as a primary culture directly from animal tissues. However, upon replication in culture, mycobactin is not an absolute requirement if the appropriate medium and starting pH are used (100).

Animal and Human Isolates

The next level of variability observed among MAP isolates from animal and human origins strikingly resembles the situation with MAV (88). Animal isolates have been linked to Johne's disease in ruminants and wildlife, and isolation from human specimens has been related to a potential etiology of Crohn's disease (CD). Restriction fragment length polymorphism (RFLP) analysis identifies three patterns, including mostly ovine (pattern A), bovine (pattern B), or caprine (pattern C) isolates (130). The sole human isolate analyzed, strain Linda, followed pattern B. Further analysis by RFLP and PCR-restriction endonuclease analysis of IS*900* and IS*1311* polymorphisms established the widely used nomenclature of C and S strains (172). C strains included most bovine isolates, isolates from wildlife, and two human isolates. Another more recent study by pulsed field gel electrophoresis combined with analysis of polymorphisms distinguished pigmented (type I) sheep isolates from nonpigmented (type II) isolates (153). Further studies by comparative DNA sequencing and representational difference analysis (a type of PCR-based subtractive DNA methodology) demonstrated an 11-bp insertion in a novel region of MAP genomic DNA (50). Comparative genomic tests failed to identify unique type-specific loci but demonstrated a greater number of deletions and rearrangements in type II strains when compared with MAV. In general, these studies indicated that MAP strains appear to cluster into two groups: slowly growing ovine strains and broad-host-range strains that include bovine, wildlife, and human isolates. As previously indicated by Stevenson et al. (153), there is no absolute correlation between strain type and host, with some ovine isolates clustering with C strains and a few bovine isolates clustering with S strains. Moreover, S strains can infect and produce Johne's disease in cattle. In another recent study, Motiwala et al. (108) used multiplex PCR of IS*900* integration loci (MPIL) and amplified fragment length polymorphism (AFLP) analyses to fingerprint MAP isolates recovered from animals (n = 203) and patients with CD (n = 7) from diverse geographic localities. Bovine isolates clustered in groups of high genetic similarity, while ovine and human isolates displayed a higher degree of variability. Moreover, the two molecular fingerprinting methods were independent, and strains that clustered in one group by MPIL could be subdivided by AFLP. The salient feature of the AFLP analysis, in contrast to other previous studies, was the higher degree of heterogeneity displayed by the human strains. Expansion of this and similar types of studies to a greater number of human isolates is of utmost importance to determine the likelihood of interspecies transmission between cattle and humans and the potential sources of MAP infection in humans (Figure 1). For example, octamer-based genome scanning identified two

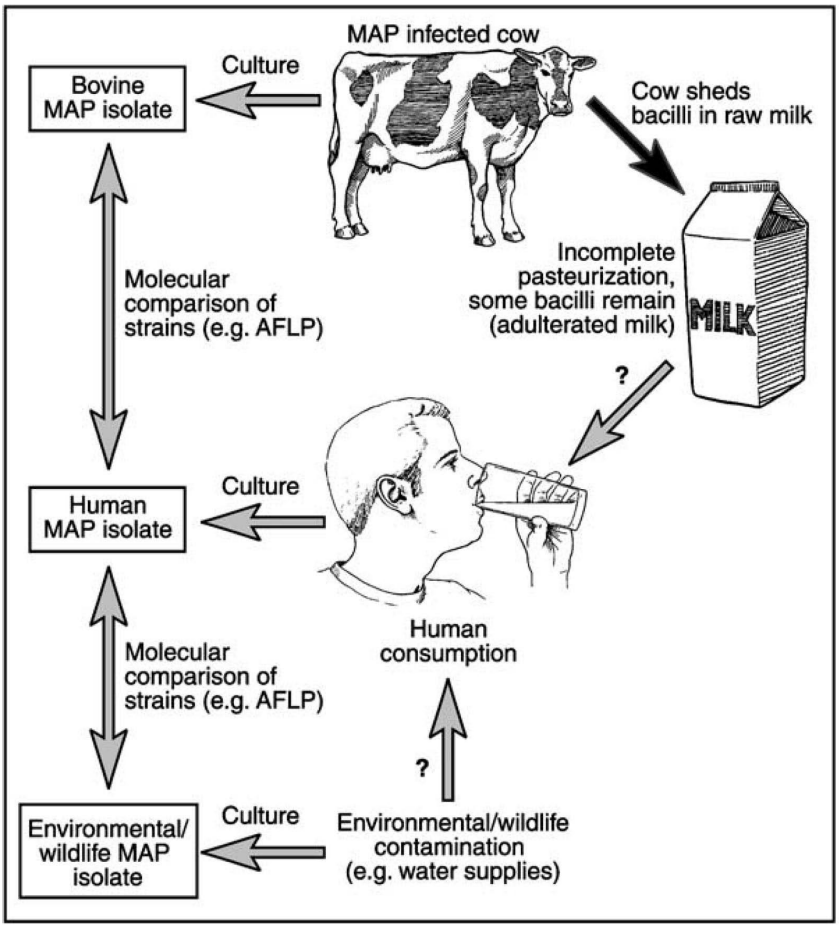

Figure 1. Potential sources of MAP infection in humans. Humans can become infected with MAP isolates found in adulterated milk or contaminated water supplies. The molecular comparison of strains by octamer-based genome scanning, multiplex PCR of integration loci, and AFLP can be used to determine the likelihood of this MAP transmission from cattle and/or the environment/wildlife to humans.

lineages of *Escherichia coli* O157:H7 that are disseminated within the U.S. cattle population (92). This analysis indicated that some patterns recovered from both cattle and humans were not randomly distributed among the lineages. This suggests that isolates recovered from cattle alone (bovine isolates) were not as virulent for humans. In contrast, the analysis of MAP strains by AFLP, though limited in the number of human isolates, does not seem to match this trend (108). If bovine MAP isolates are not readily transmitted to humans, the role of raw or adulterated milk as a source of MAP infections in CD patients would be difficult to maintain. See Bannantine et al. (6a) for a thorough review on the development of molecular methods to address concerns that MAP might be a foodborne pathogen.

Genomics

The MAP genome has recently been sequenced at the University of Minnesota, St. Paul, in collaboration with the National Animal Disease Center (NADC; Ames, Iowa). The sequencing strain (MAP K-10) was selected on the basis of our previous studies (64). This strain was provided by D. Whipple (64) as a typical bovine isolate from a cow with Johne's disease that had been subjected to a low number of passages in vitro. This would prevent loss of the virulence properties of the original isolate. In contrast, the American type culture collection (ATCC) type strain 19,698 has been passed in vitro more frequently. Moreover, our experiments demonstrated that strain K-10 yielded the highest efficiency of transformation with plasmid DNA and was readily infected by phage. Strain K-10 is a typical bovine type II strain (108). A similar situation regarding selection of transformable and virulent isolates led us to the selection of MAC 104 as the sequencing strain for MAV, whose genome sequence has now been completed at The Institute of Genomic Research (Rockville, Maryland). These properties of strain K-10 were of importance in developing a transposon mutagenesis system (74), as well as recombinant MAP strains expressing reporter genes such as those encoding the firefly luciferase (174) and the green fluorescent protein (75). These tools will be useful in postgenomic studies to create mutants in defined genes to assess their contributions to MAP virulence, thus fulfilling the molecular version of Koch's postulates. Moreover, comparative genomics and mutant analyses of both MAP and MAV would be essential for a thorough understanding of how two closely related microorganisms may cause different disease processes. The current information on the complete genomes of MAV and MAP indicates sizes of 5.48 and 4.83 Mb, respectively. The MAP genome sequence was released January 2004; see http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=41400296, accession number AE016958. However, correction of potential sequencing errors and final annotation may result in size corrections. Nonetheless, it is evident that MAP and MAV are highly homologous. For example, comparison of a 35-kb region encompassing the origins of replication of MAP and MAV showed a homology of greater than 97% (10). Moreover, this homology seems characteristic of the whole genomes as indicated by the above-cited release data. This would predict the existence of no more than 75 subspecies-specific genes, of which 21 have been located to specific sequences (6).

Both MAP and MAV genomes possess numerous insertion elements either unique to the subspecies or shared by several members of the species or the genus *Mycobacterium* (72). For example, the MAP genome has revealed the presence of 17 IS*900*, 7 IS*1311*, and 3 IS*1245* insertion elements. The IS*900* element seems unique to MAP and has been widely used as a diagnostic tool to detect MAP in clinical samples from both animals and humans. Adequate selection of primers for nested PCR of IS*900* is essential for specific amplification of this element (22, 23). IS*900*-like elements with highly homologous but not identical sequences in other mycobacteria have been observed (44, 60). The presence of IS*900* in MAV isolates from AIDS patients has also been described (115); however, these data

do not include sequencing information. Thus, under further scrutiny, these elements may be closely related, but not identical to IS*900*. For example, IS*1626*, a sequence detected in MAV, shares approximately 82% homology with IS*900* (72). Other elements present in MAP are shared, as IS*1311* is also present in *M. intracellulare* and IS*1245* is present in MAV isolates. The GS element (or IS*1612*) has been found in MAP, MAV, and the *silvaticum* subspecies. This 7-kb insertion sequence is particularly interesting because it contains genes with low GC content that may be associated with the biosynthesis, modification, and transfer of a fucose moiety to cell wall glycopeptidolipids (144). In summary, the canonical IS*900* element seems to be a defining characteristic of the MAP subspecies, though similar elements may be present in other mycobacteria. All these insertion elements may contribute to defining the pattern of gene expression in the different subspecies. For example, one study found that seven IS*900* insertion loci were conserved among all MAP strains tested and that five insertions resulted in gene inactivation (22). The genomic differences between MAP and MAV may be compounded by the effects of insertion elements on up- or downregulation of gene expression, further determining the compositions of the transcriptomes and proteomes of both types of microorganisms.

Antigens and Virulence Determinants

A major antigen of mycobacterial species including MAP is lipoarabinomannan (LAM), which has been used in many serological tests for diagnosis of Johne's disease (155). Other lipids and glycolipids of MAP have not been fully characterized, but they are expected to be similar to those from other mycobacterial species. For example, the same major polar glycopeptidolipid was found in the ATCC prototype strain 19,698 as well as in strain 18, now reclassified as MAV (26). Nonetheless, subtle differences in the glycolipids have been exploited to classify related strains of MAP and MAV (122). Serovar-specific glycopeptidolipids are not expressed by MAP or *M. avium* subsp. *silvaticum*, though the synthetic machinery seems to be present within the GS element (52).

Several immunoreactive proteins share homology with other mycobacterial antigens. These include the GroES and GroEL proteins, which are highly antigenic, and conserved heat shock proteins (40, 55). A major antigen that dominates the humoral response against MAP is the 34-kDa cell wall antigenic protein (48). This antigen has homologs in other mycobacteria and shares two identical B-cell epitopes with the MAV homolog (127). A seroreactive antigen of 34 kDa with a putative serine protease activity that is different from the 34-kDa antigen described above has also been described (25). Another conserved highly immunogenic antigen with homologs in many bacteria is bacterioferritin, also known as antigen D, with a molecular mass of 400 kDa in the oligomeric form and a subunit molecular mass of approximately 17 kDa (20). The alkyl hydroperoxide reductase proteins (AhpC and AhpD), likely involved in the detoxification of reactive nitrogen intermediates, are also abundant immunogenic proteins of MAP in sterling

contrast to their low abundance in MAV when both microorganisms are grown under various laboratory conditions (124). Other immunogenic proteins shared by mycobacteria and secreted by MAP include a 32-kDa antigen (56), a 16.7-kDa antigen, and a manganese-dependent superoxide dismutase (SodA), and proteins of the antigen 85 complex (49, 101, 110). Antigens 16.7, 85B, and SodA from MAP elicit T helper 1 (Th1)-type immune responses associated with protective immunity in mice (109–111). Protein antigens of 34.5 and 44.3 kDa have also been identified in culture filtrates of MAP by the use of monoclonal antibodies against MAP antigens, though no sequence data are available on these antigens (112). This study also observed the presence of glycoconjugates, which were the earliest antigens detected in culture filtrates.

Two antigenic proteins of importance in the context of MAP as a potential etiology of CD are the p35 and p36 antigens. Antigen p35 was identified by screening a MAP recombinant library in *E. coli* as displaying strong reactivity against sera from all clinically and a significant proportion of subclinically infected animals tested (57). No public sequence information is available on this antigen, but the corresponding gene was shown by hybridization to be homologous to DNA sequences from MAV strains and isolates from wood pigeon that likely represented *M. avium* subsp. *silvaticum*. Thus, p35 seems to be a shared antigen among all subspecies of *M. avium*. Antigen p36 was reported to be an indicator of MAP infection in relation to the seroreactivity observed in human cases of sarcoidosis and CD (57–59). With the sequence information provided for this antigen, we searched GenBank for homologous sequences. We found a significantly homologous sequence in the genomes of MAP K-10 (about 98%) and MAC 104 (about 97%). Thus, p36 also seems to be a shared antigen among the *M. avium* species. The amounts of p35 and p36 antigens in MAP strains have not been determined, and thus we do not know whether MAP strains produce larger amounts of these antigens than other subspecies of *M. avium* do.

A MAP-specific antigen is the HspX protein, a 16-kDa putative heat-shock-like protein possessing an arginyl-glycyl-aspartic acid (RGD) peptide motif implicated in mediating cell attachment and stimulating phagocytosis (53). In addition, this protein was expressed by intracellular MAP within bovine macrophages (9). As long as IS*900* appears to be MAP-specific, IS*900*-encoded proteins are also unique to the subspecies. In this context, the more abundant protein produced by this element seems to be the Hed (host-expression-dependent) protein (51). However, it is unclear whether this protein is produced in large amounts to elicit an immune response. Another candidate for a MAP-specific protein is a 42-kDa immunoreactive antigen identified by comparative two-dimensional gel analysis of methionine-labeled cellular proteins from MAP and MAV (170). However, no sequence data are available on this protein. Use of comparative genomics has led to the discovery of 21 sequences specific to MAP ranging in size from 7 to 91 kDa (7). Five of these proteins were immunogenic in mice and rabbits, and they all reacted with sera from cattle with clinical disease. This approach promises for the first time to find antigens specific to MAP that could be used in serological tests for MAP detection in animal and human hosts.

Few virulence determinants have been described. Attachment and internalization of MAP to the intestinal mucosa appear to be mediated by a fibronectin binding protein. A protein of approximately 36 kDa (FAP-P) with about 90% homology to the MAV homolog was identified in MAP strains (139). This protein seems to be made as a precursor of 54 kDa and is located in the inner part of the cell envelope rather than directly on the surface. A recombinant strain carrying an antisense construct of FAP-P demonstrated reduced expression and attachment to fibronectin (141). In addition, a 35-kDa membrane protein that reacts with antisera from cattle with Johne's disease also plays a role in the invasion of bovine epithelial cells and is upregulated by hypoxic conditions that mimic the intestinal environment (8).

Johne's Disease

The Disease in Ruminants

MAP is the etiological agent of a severe gastroenteritis in ruminants known as Johne's disease, a chronic enteritis with worldwide distribution and a significant impact on the world economy (156). In the United States, losses to the cattle industry have been estimated at $1.5 billion per year (147). Lower estimates have been given, but since it is difficult to assess losses in productivity and profit at an individual herd level, the impact of this disease may be underestimated nationwide (89, 128). A regression model estimates these losses to be from $40 to $227 per cow inventoried per year (114).

The physiology and pathogenesis of MAP have been reviewed recently (72). Neonates and juvenile animals are the most susceptible to infections (38). Disease progression is classified in four stages: silent infection, subclinical, clinical, and advanced clinical disease (171). Animals are most likely infected via the fecal-oral route. MAP colonizes the mucosa-associated lymphoid tissues of the upper gastrointestinal tract, which are endocytosed by the M-cells of the ileal Peyer's patches. Bacilli are subsequently phagocytosed by subepithelial and intraepithelial macrophages (66, 103, 107). The antigen FAP-P may play a role in the binding to fibronectin that would facilitate endocytosis mediated by surface integrins of the apical surface of M-cells (139). Once internalized, intracellular bacilli remain and multiply in the phagosomal compartment (90). The ensuing host cellular immune response leads to granuloma formation and the involvement of adjacent lymph nodes (38, 103). T-cell activation, gamma interferon (IFN-γ) production, and macrophage activation take place, and this process leads to the clinical manifestations characterized by a corrugated intestinal epithelium. The final consequence is the appearance of the typical malnutrition syndrome associated with Johne's disease that culminates in the death of the animal.

The Koch's Postulates: Application to Johne's Disease

Koch's postulates represented a landmark development for establishing the etiology of infectious diseases. Koch applied his postulates to establish unequivocally that *M. tuberculosis* is the etiologic agent of human tuberculosis (95). Though skin tests have revealed that almost one third of the world's population is infected (154a), only a significant but minor proportion of infected individuals develop active disease. In the acute phase, acid-fast bacilli are evident in diseased individuals and the microorganisms can be readily isolated in pure culture. Moreover, isolated bacilli are quite lethal to a variety of animals and can be readily reisolated from infected tissues of experimentally infected hosts. However, Koch's postulates may not be applied without reservations to any host-pathogen interaction. The case of *M. leprae*, the causative agent of leprosy, clearly illustrates this issue. This microorganism has not yet been cultured in vitro, but causation has been established by a combination of immunological and chemotherapeutic approaches.

In the case of Johne's disease in ruminants, the application of Koch's postulates and the establishment of MAP as the causative agent were facilitated by a serendipitous finding. An extract of *Mycobacterium phlei* allowed in vitro growth of the microorganisms present in animal tissues by providing mycobactin, a necessary nutrient for MAP in the iron-deficient conditions used for those cultures (163). Figure 2 summarizes the historical developments associated with the fulfillment of the Koch's postulates for MAP and Johne's disease. A more detailed description of the history of the disease is presented elsewhere (31), but the following narrative follows the history in connection with the Koch's postulates. Our story starts in a German farm with a cow that failed to gain weight until the animal died. In 1895, Johne and Frothingham analyzed the intestines and identified acid-fast bacilli, establishing that bacilli could be isolated from infected animals. Koch's postulates require growing the agent in pure culture and this was accomplished by F. Twort, who isolated the bacillus in 1910. The final test of the postulates was to reintroduce the bacilli in susceptible animals and show that laboratory animals developed a comparable disease. Twort accomplished this aspect in 1914 (162). However, the animal model plays a critical role at this final stage and it is often difficult to draw conclusions that will apply to the homologous host. The postulates were fulfilled in 1933, when Hagan & Zeissig reported in their six-year study that a herd had been experimentally infected with MAP (70). Since then, the establishment of the Koch's postulates for Johne's disease has led control and eradication efforts.

Significant research efforts are directed toward the identification of genes and their products that may be involved in the pathogenesis of Johne's disease. In this context, our goal is to fulfill a molecular version of the Koch's postulates (62). Figure 3 depicts an in vitro version using bovine macrophages. Individual transposon mutants of MAP are tested for survival in bovine macrophages, and mutant strains with impaired survival are identified. Molecular procedures are then used to identify the mutated genes and the wild-type homologs are reintroduced back into the mutant strains with the expectation that macrophage survival is restored. For in vivo experiments, strains are then tested in small-animal models and ruminants.

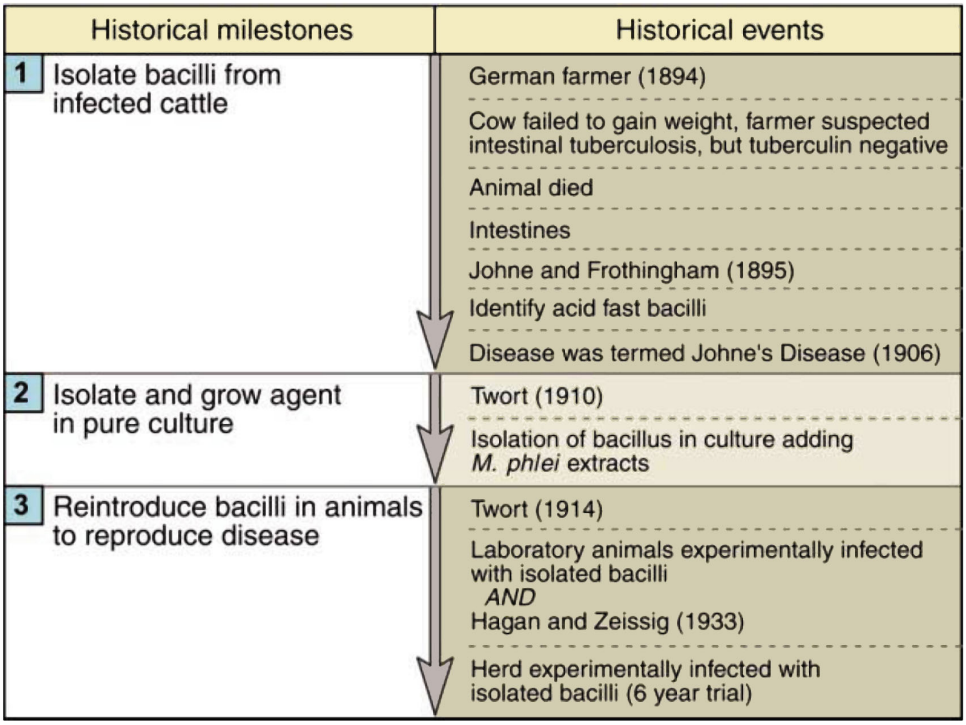

Figure 2. Historical milestones associated with Koch's postulates. In ruminants, the etiologic agent of Johne's disease was established as MAP by applying Koch's postulates (*left*). Historical events (*right*) indicate how the various postulates were fulfilled.

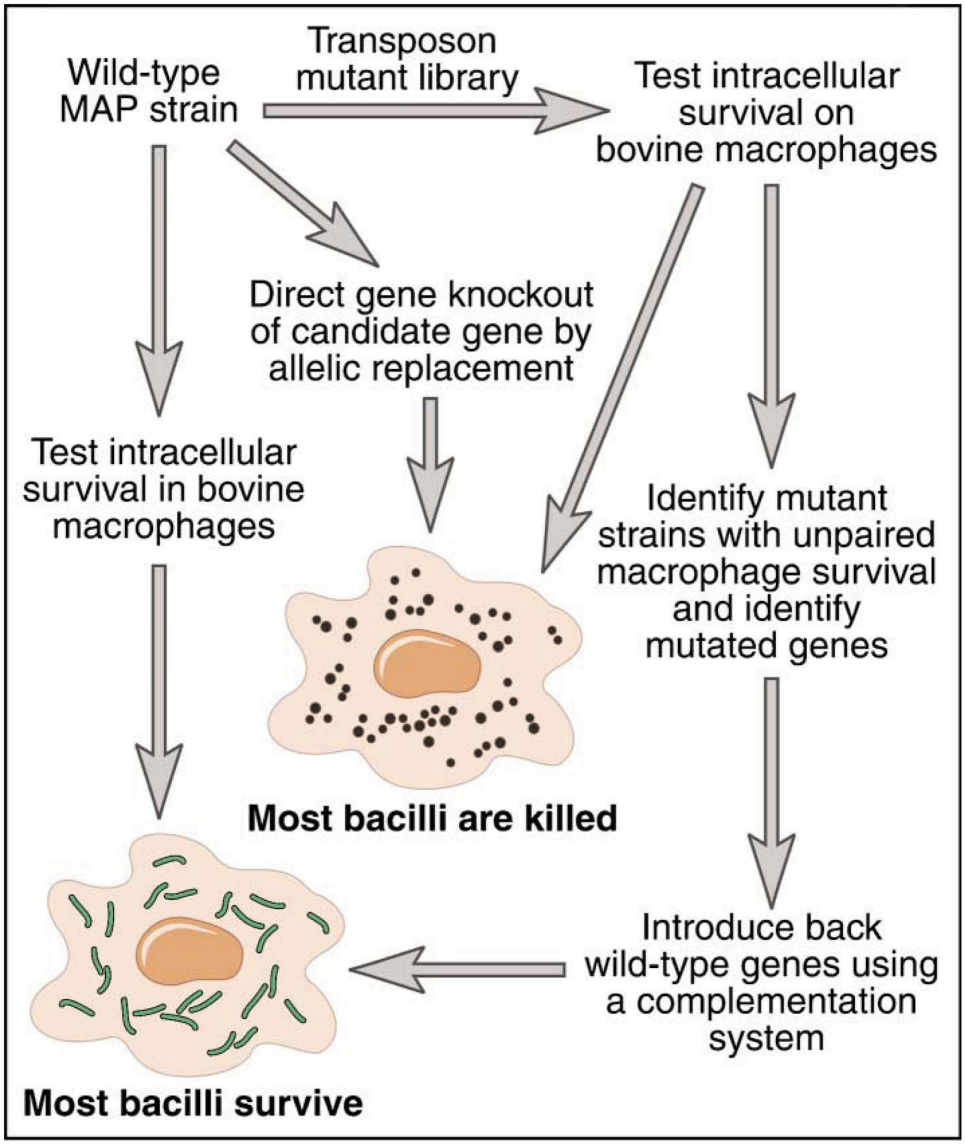

Figure 3. A molecular version of Koch's postulates. Transposon mutants of MAP are tested in vitro for survival in bovine macrophages. Molecular procedures identify genes and their products involved in the pathogenesis of Johne's disease.

Animal Reservoirs and Diagnostics from Clinical Samples

MAP is a microorganism with a wide host range, and infections have been reported not only in ruminants but also across many species including badger, bison, camelids, deer, elk, fox, primates, rabbits, and swine. Particularly interesting are two cases in primates that resemble Johne's disease in ruminants. These findings seem to indicate that nonruminant wildlife may be a natural reservoir for MAP (17a). Three interesting studies performed in Scotland underscore the role of rabbits in the transmission of paratuberculosis (12, 13, 69). These studies indicated a correlation between the number of infected rabbits and infected cattle. This opens the possibility that cattle may consume pastures with fecal pellets from infected rabbits and thus acquire MAP. Moreover, MAP strains isolated from rabbits in these farms are closely related to cattle strains and produce disease in experimentally infected cattle. Badgers and foxes consume rabbits as food, and thus, these carnivores are likely to be infected with MAP by this food chain. The infection cycle may be completed by transmission of environmental MAP from water supplies, whose pathogenicity may be enhanced by intracellular multiplication within protozoans as shown for MAV (37). It can then be speculated that environmental MAP carried in protozoan hosts are the source of rabbit infections, with these animals transmitting MAP to cattle and even carnivores through the food chain.

Methods to diagnose MAP from clinical samples have been reviewed elsewhere (42, 72). In general, methods can be divided into those that detect the microorganism or its DNA, and those that look for a specific host reaction against MAP antigens. All methods may be then compared with the "gold standard" of culturing the microorganism from fecal samples. The most widely used PCR method is based on the detection of the IS*900* element. The immune-response-based methods include a skin test, an INF-γ-ELISA test for cellular immunity, and the widely used ELISA-LAM or ELISA-protoplasmic antigen humoral tests. With some caveats, these methods have high specificity but lack sensitivity to detect subclinically infected animals. INF-γ-ELISA becomes positive earlier during disease progression, whereas the humoral tests become positive at a later stage. Likewise, fecal culture is negative for afflicted animals that shed a low number of bacilli, and ovine strains need specially modified medium for culture (173). The use of two to three different tests or serial testing is recommended, but none of the diagnostic tests are capable of detecting all subclinically infected animals.

Pathological and Immunopathological Findings in Diseased Animals

The pathology of paratuberculosis is characterized by chronic enteritis, chronic lymphangitis, or mesenteric lymphadenopathy (21). Granulomatous lesions may be patchy, mild, or severe, and are usually localized in the distal intestine, the ileocecal lymph nodes, or the adjacent area of the colon beyond the ileocecal valve. Infiltrates of lymphocytes, macrophages, and plasma cells are observed and are

either diffused or focalized according to the severity of the infection becoming transmural (throughout the entire thickness of the wall). Extraintestinal cases in liver or kidneys also occur (79). However, affection of the villi of the mucosal epithelium of the ileum is the major component of the pathogenesis resulting in the malabsorption syndrome (see below for a comparison with human inflammatory bowel disease).

The immunopathology of paratuberculosis stems from MAP being a facultative intracellular pathogen. In this context, the ability of MAP to survive within macrophages plays a critical role in disease development (72). It appears that intracellular multiplication and killing occur simultaneously, reflecting an initial Th1 cellular immune response of the host that switches to Th2 in animals with clinical disease (177). This T-cell response may be pivotal in activating intestinal macrophages, as mice deficient in T-cells have a marked reduction in the number of activated macrophages (159). Nutritional and hormonal factors influence host susceptibility. Reduced dietary calcium protects beige mice from MAP infections (149), but a corresponding increase in endogenous vitamin D reverses the beneficial effects of low calcium (148, 149). Transient exposure of monocytes to growth hormone or prolactin enhances intracellular multiplication in primary bovine monocytes (63).

Mycobacteria are relatively resistant to the bactericidal mechanisms of professional phagocytes. Catalase and peroxidase are involved in resistance to reactive oxygen intermediates (28, 104), and AhpC, a major antigen of MAP (124), may be important to protect against both oxygen and nitrogen reactive intermediates (29, 104). Likewise, the secreted antigen 85B elicits high production of IFN-γ, nitric oxide (NO), and interleukin (IL)-10 in spleen cells of immunized mice (109). In contrast, stimulation with SodA increases production of IFN-γ, but lowers NO and IL-10 production (111). Furthermore, the host T-cell population may influence the production of reactive intermediates by professional phagocytes, as athymic nude mice produce significantly less inducible NO synthase (159). Treatment of MAP-infected bovine monocytes with IFN-γ induces release of NO, but the levels seem insufficient to kill MAP in vitro (176). Similarly, growth of MAP in the J774 murine macrophage cell line can be influenced by prior exposure of the macrophages to different levels of tumor necrosis factor alpha (TNF-α) (146). Moreover, activation of J774 macrophages by IFN-γ or lipopolysaccharide (LPS) leads to increased colocalization of MAP with acidic late endosomal compartments, suggesting a more efficient killing of intracellular bacilli (81).

The exact mechanism of how MAP influences normal antigen processing in macrophages is unknown. In murine cultured macrophages from the J774 cell line, MAP seems to undergo a growth phase during the first 24 h postinfection, followed by a slow decline in viability for the next six days (4). On the basis of the selective exclusion of specific subcellular markers in J774 macrophages, other studies have shown that both MAP and MAV can persist within phagosomal compartments for up to 15 days (98). MAP, MAV, and *M. tuberculosis* all appear to inhibit phagolysosomal fusion (39, 121) and acidification (98, 121). However,

because the interaction between the ruminant immune system and MAP may not follow similar interactions between human and murine host cells and other mycobacteria, the use of these murine or human models and cell lines may not accurately reflect the infection in the ruminant host. For example, after infection of J774 macrophages with both MAP and MAV, numbers of intracellular MAP decreased while MAV increased over a four-day period (98). Recent studies with a bovine macrophage cell line demonstrated that patterns of gene expression in macrophages are different upon phagocytosis of latex beads, *E. coli*, or MAP (161). Likewise, primary bovine monocytes react differently to MAV or MAP infections, since only the latter seems to induce a suppression of macrophage activation mediated by oversecretion of IL-10 (169). In studies with primary bovine macrophages, live not dead MAP stimulates apoptosis (4). Moreover, stimulation of monocytes from MAP-infected cattle leads to downregulation of gene expression in clinically infected animals and upregulation in subclinically infected cattle (46). In addition, infection with MAP appears to reprogram the pattern of gene expression of bovine macrophages (45). A recent study showed that whole blood from infected cattle in the subclinical stage had a lower expression of TNF-α, RANTES, and monocyte chemoattractant protein 1 compared with that from uninfected cattle (24). Overexpression of IL-10 may mediate this effect. The outcome of this state of immunosuppression would be to weaken protective immunity, reduce granuloma formation, and perpetuate the infection.

Genetic Basis of Resistance and Susceptibility

Host genetic factors influence the degree of susceptibility to infectious diseases. Use of recombinant mouse strains led to the identification of a dominant gene on chromosome 1 encoding the integral membrane protein NRAMP1 (natural resistance associated macrophage protein 1). This protein is expressed in macrophages and is involved in transport of divalent cations (68). In addition, alleles of NRAMP1 in the mouse were associated with natural resistance to brucellosis, leishmaniasis, and tuberculosis, diseases caused by intracellular pathogens subverting macrophage defenses. These findings were also applicable to other intracellular pathogens such as MAP. Homologs of the NRAMP1 gene are found in cattle and humans. Polymorphisms in NRAMP1 were analyzed in cattle naturally susceptible or resistant to tuberculosis, but no association between these two parameters was found (11). Thus, at least in cattle, it seems that another locus or loci may control the resistance/susceptible phenotype. In humans, some correlation has been found between NRAMP polymorphisms and tuberculosis susceptibility (14, 93), but this association is stronger in mice. The ongoing project on the sequencing of the bovine genome at the Baylor College of Medicine (Houston, Texas) opens exciting possibilities to the discovery of disease-resistant genes in cattle: http://hgsc.bcm.tmc.edu/projects/bovine. Comparative genomic approaches may then be applied to characterize disease-resistant genes across the various dairy and beef breeds and determine whether any would have greater natural resistance to MAP infection.

Inflammatory Bowel Disease

Crohn's Disease and Ulcerative Colitis

Inflammatory bowel disease (IBD) is a general term that describes a group of chronic gastrointestinal inflammatory disorders of unknown or still unproven etiology. The disease was originally described by Crohn, Ginzburg, and Oppenheimer in 1932 (47). IBD affects millions of people worldwide; and in the United States alone, the Crohn's and Colitis Foundation of America has estimated that there are about one million individuals affected. IBD may affect male and female adults and children equally, and it has a peak age at onset between 15 and 25 years of age. A lesser peak age at onset exists between 55 and 65 years of age. Moreover, IBD is on the rise in northern Europe and North America (151). Disease prevalence is higher in urban rather than rural areas and in some populations, such as in Ashkenazi Jews (65a). IBD can be divided in two major groups, ulcerative colitis (UC) and Crohn's disease (CD). UC is characterized by diffuse mucosal inflammation that involves the rectum and may extend in continuity, without areas of intact mucosa, to all or part of the colon. In 10 to 20% of patients with total colitis, the inflammatory process may extend into the terminal ileum. CD is characterized by transmural inflammation that involves any segment of the gastrointestinal tract from the mouth to the anus, although it often spares the rectum (Figure 4). It may also involve the liver and the pancreas. When the disease affects the small intestine, it is also known as regional enteritis. In contrast with UC, CD is segmental, with spared areas of normal mucosa surrounded by diseased tissue, a characteristic that gives the disease a macroscopic patchy or "cobblestone" appearance. Extraintestinal symptoms are also associated with IBD and are more common in patients with perianal CD. They include, among others, dermatologic, rheumatologic, ocular, hepatobiliary, and urologic manifestations (65a, 104a, 178).

In summary, diarrhea is one of the major symptoms in the clinical presentations of IBD. In UC, it is usually accompanied by rectal bleeding, mucus, fecal urgency, tenesmus, and left lower quadrant cramps relieved by defecation. Mild or moderate disease may be accompanied by low-grade fever, anemia, and hypoalbuminemia. Severe disease is characterized by more than six bloody bowel movements per day, leading to anemia, hypovolemia, tachycardia, and hypoalbuminemia. Abdominal pain and tenderness are usually present. Extracolonic manifestations such as erythema nodosum, pyoderma gangrenosum, episcleritis, thromboembolia, and oligoarticular arthritis are associated with active disease. In contrast, uveitis, ankylosing spondylitis, and sclerosing cholangitis may be present independent of colitis activity. In CD, chronic diarrhea is usually accompanied by fever, abdominal pain, anorexia, weight loss (of up to 10 to 20% of body weight), and a right lower quadrant mass or fullness. In some patients, the first signs of the disease may be acute abdomen or intestinal obstruction. Inflammation in CD may result in either a fibrostenotic-obstructing pattern or a penetrating-fistulous pattern of disease. Diarrhea is usually nonbloody and intermittent. A mass may be palpable in the right lower quadrant of the abdomen, and

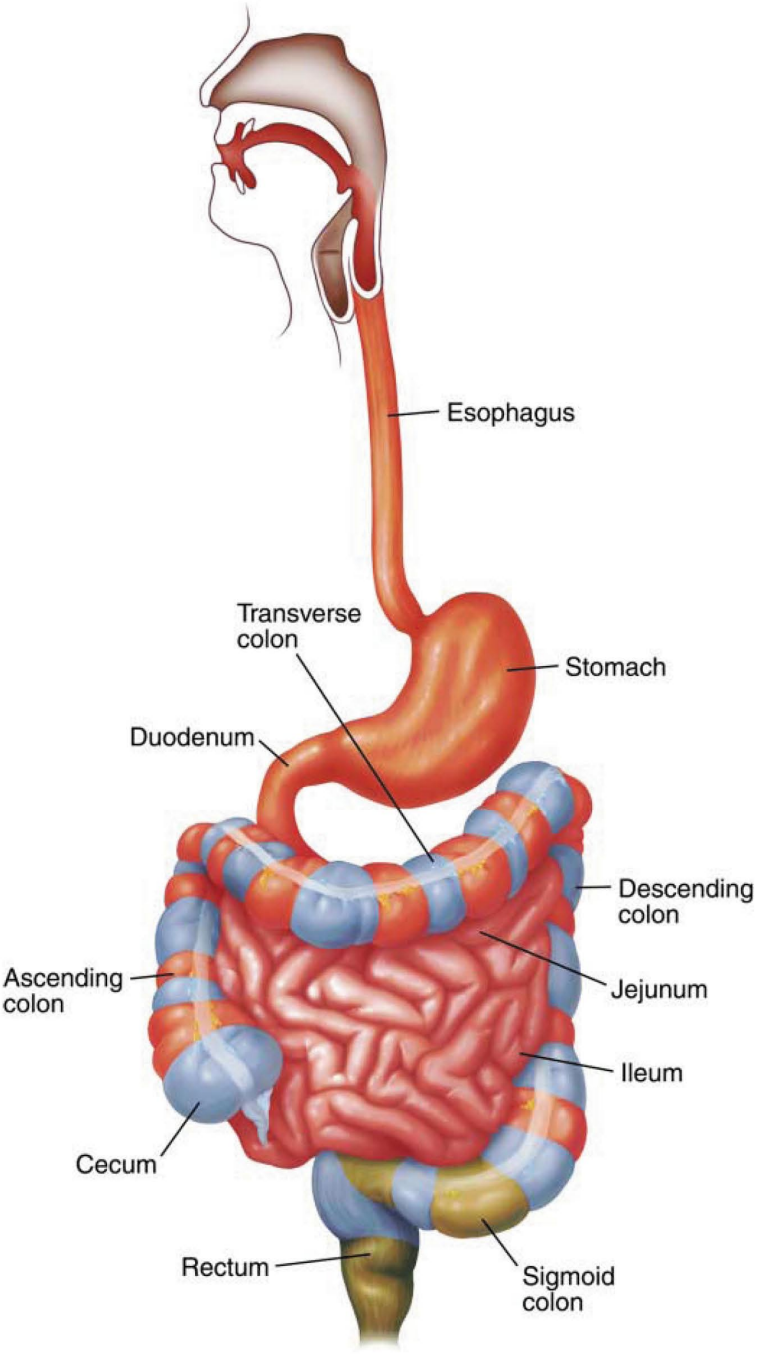

Figure 4. Anatomical regions affected by IBD. UC (*green*) is characterized by mucosal inflammation of the rectum and colon. CD (*red*) has transmural inflammation of any gastrointestinal tract segment except the rectum. The areas shaded in blue and red indicate locations where both UC and CD may be encountered. The areas shaded in blue and green indicate locations where UC predominates and CD is less frequent.

it consists of inflamed bowel, adherent and indurated mesentery, and enlarged lymph nodes. Edema, wall thickening, and fibrosis within the mass may lead to a narrowed intestinal lumen. The early stages of ileocolitis are characterized by intermittent obstruction and postprandial pain followed, after several years, by fibrostenotic narrowing and strictures. Diarrhea will then be less frequent, being replaced by chronic bowel obstruction with some acute episodes and constipation. Inflammation of the ileocecal region may lead to wall thinning and microperforation, with fistula formation.

Malabsorption and steatorrhea are common in patients with jejunoileitis. In these patients, bacterial overgrowth in obstructions or fistulas, bile-acid malabsorption, and intestinal inflammation during active disease may cause diarrhea. Nutritional deficiencies and intestinal malabsorption may result in a wide variety of systemic manifestations, including vertebral fractures, pellagra, and megaloblastic anemia. Patients with colitis may present low-grade fever, abdominal cramps, malaise, and diarrhea. Severe inflammation may result in toxic megacolon. If stricture occurs, symptoms of bowel obstruction may be present. Vomiting may be present when fistulization occurs into the stomach or duodenum. If fistulization occurs into the proximal or mid-small bowel, it may result in malabsorption. Rectovaginal fistulas may be also present. One third of patients with large or small bowel involvement develop perianal disease, with anal fissures, perianal abscesses, large hemorrhoid tags, and fistulas. Nausea, vomiting, and epigastric pain suggest gastroduodenal involvement in CD. Severe disease may result in chronic gastric outlet obstruction. Extraintestinal manifestations of CD include those described for UC. Additionally, oral aphtous lesions, gallstones, and nephrolitiasis may occur (65a, 104a, 178).

Pathological and Immunopathological Findings in Inflammatory Bowel Disease

Pathological and immunopathological findings in IBD are described elsewhere (65a, 104a). The Gastroenterology and Hepatology Resource Center at The John Hopkins Medical Institutions (Baltimore, Maryland) has further illustrative examples and guided easy-to-follow descriptions: http://www.hopkins-gi.org/pages/latin/templates/. In UC, there is an inflammatory reaction primarily involving the colonic mucosa. Grossly, the colon appears ulcerated, hyperemic, and unusually hemorrhagic. A characteristic aspect of the inflammation is that it is uniform and continuous, with no areas of normal mucosa present. The rectum is involved in 95% of the cases. The surface mucosal cells, as well as the crypt epithelium and submucosa, are involved in the inflammatory reaction, with infiltration of neutrophils. This picture progresses to epithelial damage with loss of surface epithelial cells resulting in multiple ulcerations. Infiltration of the crypts by neutrophils often results in characteristic small crypt abscesses and their eventual destruction. Loss of the crypt epithelium, loss of goblet cells, and submucosal edema may occur. Following frequent cycles of inflammation, mild submucosal fibrosis can develop. Unlike CD, deeper layers of the bowel beneath the submu-

cosa are not usually involved. In severe cases of UC, the bowel wall may become extremely thin, leading to possible dilatation and subsequent perforation. Recurrent inflammation leads to characteristic features of cronicity, such as fibrosis and retraction. As a consequence, the colon becomes shortened and "smooth." In prolonged cases of UC, the surface epithelium may show features of dysplasia. Significant dysplasia in colonic biopsies is associated with significant risk of coexistent carcinoma.

In contrast, CD is characterized by chronic inflammation extending through all layers of the intestinal wall and involving the mesenteric as well as regional lymph nodes. This basic process occurs in any place of the small or large intestine. One of the most distinctive macroscopic features of CD is the sharp demarcation of the segmental bowel involvement, which may occur in any level of the intestine. Initially, CD is undefined, with the ileum appearance being usually hyperemic and the mesenteric lymph nodes swollen. At this stage of the disease the bowel wall is only edematous. Approximately 80% of patients with this presentation are infected with *Yersinia enterocolitica*, an organism capable of producing limited inflammatory ileitis. The most characteristic histologic features of CD are transmural inflammation (affecting all the layers of the serosa), noncaseating granulomas (resembling those of sarcoidosis), dilatation of the lymphatic channels, and lymphoid aggregates in all levels of the intestines. Granulomas are the most helpful microscopic feature to distinguish CD from other forms of IBD. As the disease progresses, the gross appearance becomes characteristic. The bowel wall becomes thickened and the lumen narrows. This stenosis, typical of the disease, can occur in any portion of the intestine and may be associated with varying degrees of obstruction. The mesentery appears greatly thickened and fatty and often extends over the serosal surface of the bowel. The mucosa can be normal or have minor manifestation of disease, in contrast to that observed in UC. The disease is usually discontinuous, with areas of normal bowel separated from each other by areas of severe disease. A large percentage of patients do not have disease in the rectum, in sharp contrast to those with UC.

Another important aspect of the potential role of MAP in IBD disease is the pathological resemblance between Johne's disease and CD (Figure 5). Both are transmural diseases, though the transmural condition is rare in Johne's disease. There are also important differences, most notably the absence of fibrosis, fissures, fistulas, and pseudopolyps in Johne's disease, features usually found in CD (17a). In contrast, UC does not display transmural alterations.

Possible Etiologies of Inflammatory Bowel Disease in Humans

The etiology of IBD has not been defined. The current consensus hypothesis is that in genetically predisposed individuals (as suggested by epidemiological and genetic data) exogenous factors (e.g., infectious agents, normal flora) and host factors (e.g., intestinal epithelium, vascular supply, hormones, neuronal activity) act together to cause and maintain a chronic state of dysregulated mucosal im-

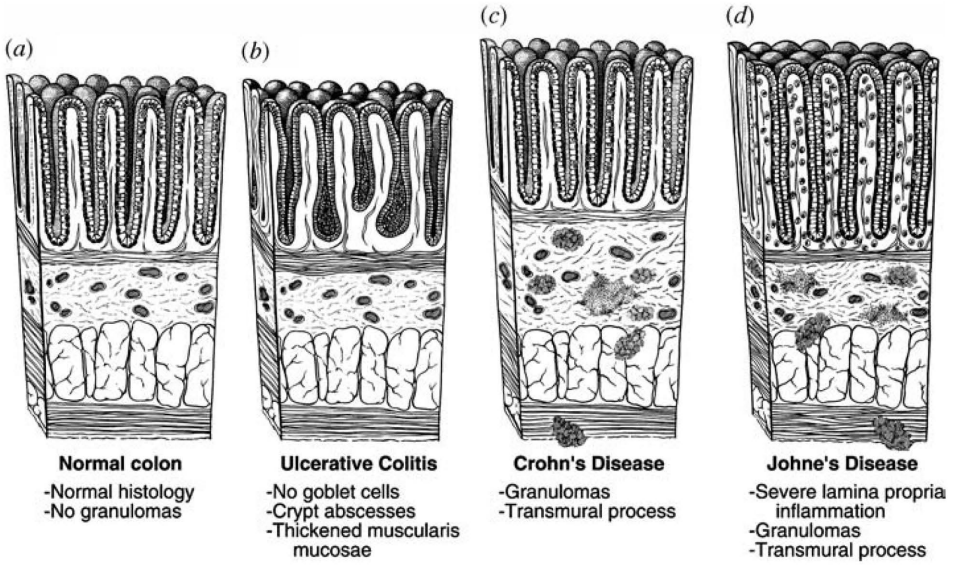

Figure 5. Colon histology of humans and animals with normal and inflammatory conditions. (*a*) Normal histology of the human colon with no granulomatous lesions. (*b*) Pathology of the human colon in UC showing a thickened muscularis mucosae and additional alterations confined to the lamina propria without transmural involvement. (*c*) Pathology of the human colon in CD showing granulomas and transmural involvement. (*d*) Pathology of the bovine colon in a severe case of Johne's disease showing lamina propria inflammation, granulomas, and a transmural process.

mune function that may be affected by specific environmental factors (e.g., smoking, chemicals). A chronic infectious agent may be responsible for the sustained activation of the mucosal immune system. However, such an agent has not yet been identified. Hence, IBD is currently considered the result of an inappropriate immune response with or without some autoimmunity component (65a).

THE NONINFECTIOUS ETIOLOGY HYPOTHESIS

The theory that an immune mechanism may be involved is based on the concept that the extraintestinal manifestations that may accompany these disorders may represent autoimmune phenomena. Thus, therapeutic agents are active because of their effects on the immune mechanisms. Furthermore, through the study of patient and mouse models it has emerged that CD is driven by the production of IL-12 and IFN-γ in a type of Th1 response (123). In contrast, UC is probably driven by the production of IL-13 (97). More recently, IL-18 polymorphism has been associated with increase risk of CD (157). In general, the transcripts to proinflammatory cytokines are elevated in CD, possibly representing disease activity. In this case, the measure of cytokines can be used as an indication of active disease and as a marker for response to therapy. Patients with CD may have antibodies to co-

lon cells, bacterial antigens (such as LPS), and foreign antigens (such as cow milk proteins) (165). These findings may be explained by the release of intestinal cell antigens. Immune complexes have also been invoked to explain extraintestinal manifestations of IBDs, such as arthritis (80). Cell-mediated immunological damage is an attractive hypothesis because of the large number of macrophages or lymphocytes in the lesions. Associated abnormalities of cell-mediated immunity include anergy and decrease of peripheral T-cells (166). Moreover, dendritic cells (DC) may play an important role in this abnormal T-cell response because CD patients demonstrate elevated numbers of DC-SIGN$^+$ (DC-specific intercellular adhesion molecule-grabbing nonintegrin) and CD83$^+$ DC in colon tissues (158a). The involvement of the lectin-receptor DC-SIGN may also be relevant to the infectious hypothesis (see below) because some pathogens, including *M. tuberculosis*, bind DC-SIGN to subvert the normal antigen processing pathway (66a).

THE INFECTIOUS ETIOLOGY HYPOTHESIS: THE CASE FOR *M. paratuberculosis*

The chronic inflammatory nature of these diseases has prompted the search for a possible infectious etiology. Despite the numerous attempts to find known infectious agents, none have been isolated. Microorganisms including *Helicobacter pylori* (131), MAP, *Pseudomonas* spp., and *Yersinia* spp. have been associated with the etiology of IBD (30, 89a). In addition, the Epstein-Barr virus and other cytophatic RNA intestinal viruses have been considered candidate etiologic agents (17, 89a). Several factors may be considered in the study of infectious causality of IBD. These factors may range from those described in the classical Koch's postulates to establish etiology of infectious diseases to those including state-of-the-art molecular Koch's postulates to identify virulence determinants (62, 95). In addition, some limitations of the Koch' postulates must be considered when establishing disease causation, especially for cases in which the pathogen is difficult to isolate or culture. For these cases, new criteria of causality based on the application of nucleic acid sequence-based identification of microbial pathogens may be applied (65). In addition, Hill-Evans postulates based on strength, consistency, specificity, and temporality of agent-disease association may be helpful (61, 77). In this context, IBD does not seem amenable to the application of the classical Koch's postulates. Although none of these factors are sufficient to prove or discard causality, their use may be helpful in narrowing a list of possible causative agents. The candidate agents can then be targets of further studies aimed to gather enough evidence to sustain a case for causality. A recent application of Hill-Evans postulates to the MAP-CD etiological linkage did not reach a definitive conclusion, underlying the difficulties involved in proving causality (17a).

The hypothesis that MAP could be the etiologic agent of CD was proposed years ago following the characterization of mycobactin-dependent mycobacteria isolated from resected gastrointestinal tissues of a few CD patients (32, 34). These isolates were pathogenic for mice and other laboratory animals, and most significantly they produced granulomatous disease of the distal intestine in an experimentally infected baby goat (34). These findings were suggestive but fell short of

fulfilling the Koch's postulates, since it is disease equivalence in animals and humans that is precisely in question. In addition, MAP isolates cannot always be recovered by culturing human specimens. An explanation for this difficulty came from further microscopic studies revealing the possible presence of spheroplastic or cell wall–deficient (CWD) forms, also known as L-forms. Molecular methods were then used to identify these aberrant acid-fast negative forms as MAP (33, 167). This led to the MAP-CWD hypothesis for the etiology of CD: Low numbers of CWD forms of MAP, difficult to detect and isolate, are the causal agents of at least some cases of CD (30).

The existence of CWD forms in CD tissues could not be taken as factual because the evidence presented was preliminary and artifacts could not be ruled out. Protocols were later developed for the identification of CWD forms on the basis of in situ hybridization in tissue samples from animals and CD patients (84–86, 138a). In one case, 35 of 48 tissue samples of CD patients were positive for the presence of MAP within granulomas (138a). These methods are based on the use of low concentrations of proteinase K to disrupt tissue samples and detect chemically generated CWD forms, and yielded positive results with tissue samples. Competent acid-fast bacilli are not detected by these methods. Nonetheless, these procedures are based on the detection of DNA and do not definitely prove that viable CWD forms are detected in these tissues. Nonviable, partially or completely lysed cells may yield similar results. More recently, less disruptive methods such as confocal scanning laser microscopy (118) have been used in the search for CWD forms or competent bacilli in CD tissues. This technology has been applied to a few tissue samples, and more definitive conclusions await a more comprehensive study. An elegant recent study generated CWD forms chemically by growing MAP in medium containing glycine followed by lysozyme treatment (78). CWD forms remained viable and reverted to normal bacilli once the treatment was stopped. CWD forms did not display bands associated with cell wall glycolipids including lipoarabinomannan. Most importantly, sera from cattle with Johne's disease reacted differently to CWD and competent bacilli, with CWD forms lacking the 21- to 30-kDa immunodominant band observed normally. Nonetheless, it is not yet known whether these chemically generated forms are infectious or whether they are equivalent to CWD forms that might be generated in vivo.

Culturing from humans proceeded through the years and a significant number of MAP human isolates have been now cultured. More recently, the application of short-term cultures using the mycobacterial growth indicator tube (MGIT) system yielded positive cultures in 6 of 7 resected tissue samples and in 4 of 20 biopsy specimens obtained from CD patients (138). Positive cultures in resected tissue (closer to the active ulcer) were obtained in 10 to 12 weeks, while up to 40 weeks were required for cultures from biopsies. Non-IBD controls yielded an overall 6% recovery of MAP-positive culture from sample specimens versus 37% recovery from CD patients. Moreover, there is one report that indicates positive MAP cultures from breast milk of lactating mothers with CD, a finding of significant importance if confirmed by other laboratories (117).

PCR technology for the detection of nucleic acid sequences has found MAP in intestinal tissues of CD patients, as reviewed elsewhere (76). A recent study used five methods for detection of MAP, adapted from diagnosis procedures for Johne's disease, and found a higher level of MAP detection in CD and UC patients when compared with controls (43). However, the MAP etiology theory has remained controversial because of conflicting results and differences in laboratory techniques among the various studies. In this context, recent observations (16) as well as previous studies have reported either positive or negative PCR test results for the identification of MAP from tissues of CD patients (72). Nonetheless, investigators who obtained positive tests have indicated that sample selection and processing is of utmost importance to detect MAP DNA by PCR in material from human intestinal biopsies. When this methodology was used with primers uniquely specific to the IS*900* element, MAP was detected in 92% of CD cases compared with 26% of non-IBD controls (23). Moreover, in this study, the use of MGIT led to a high degree of culture-positive IBD samples (14 of 33 CD cases versus 3 of 33 non-IBD controls by PCR of MGIT cultures). Another approach used to detect the presence of viable MAP in CD patients was RT-PCR, which probes for the presence of RNA. In one study, eight CD, two UC, and two non-IBD controls samples of resected ileal mucosa were subjected to RT-PCR, yielding positive results for all the IBD samples (105). Nonetheless, finding MAP in CD patients is a proof of association rather than causality, and thus this theory has not yet been accepted. In addition, questions on the reproducibility of the various studies have loomed in the background.

Genetic Factors Underlying Resistance and Susceptibility

IBD occurs more often in some racial groups and exhibits some familial clustering. This suggests that there may be a genetic predisposition to the development of the disease. In twin studies, 20% and 67% of monozygotic and 0% and 8% of dizygotic twins were concordant for UC and CD, respectively (164). Further indication of genetic predisposition is the association of IBD with several genetic syndromes, including Turner's syndrome (5). Hermansky-Pudlak syndrome, an autosomal recessive disorder, is associated with a granulomatous colitis (137). Crohn's-like lesions may appear in the large and small intestine of patients with the autosomal recessive glycogen storage disease type 1b (136). Several immunodeficiency disorders, including hereditary angioedema, hypoglammaglobulinemia, and selective immunoglobulin A (IgA) deficiency are also associated with IBD (87).

Several chromosomal regions implicated in the predisposition to IBD have been identified. The chromosomes 16q, 12q, 1p, 1q, 3p, 3q, 5p, 6p, 7q, 14q, and 19p may be specifically associated with CD (35). The locus on the chromosome 16q 12 (IBD1 locus) has been associated with CD (83), and other loci such as the locus IBD2 on chromosome 12q contribute to IBD in general. These findings suggest that the increase in the risk of intestinal inflammation is linked to specific

genes. The susceptibility gene located on chromosome 16 is the NOD2 (nucleotide oligomerization domain), which codes for a protein involved in the initial response of the immune system to bacterial infection (82, 119). This protein contains two C-terminal caspase recruitment domains (CARDs) and is also called CARD15. In the case of NOD2/CARD15, self-binding results in activation of the protein, which contains a leucine-rich region that is critical for mediating the interaction with bacterial polysaccharides (36). No correlation has been observed between these variants and UC. In subjects carrying one copy of any of these genes, the risk of developing the disease increases from 1.5- to 4-fold. Homozygous individuals had an increased risk of 15- to 40-fold. NOD2 has been identified in Paneth cells of the intestinal tract, which suggests a role for NOD2 in the regulation of Paneth cell's mediated response against intestinal bacteria. In addition, this finding may be a plausible explanation for the selective association of NOD2 mutations with ileal disease. The impaired capacity of patients with CD-associated mutations to sense bacteria in the lumen may result in increased susceptibility to certain gut microbes (120). Because of the proposed role of NOD2 to directly bind bacterial LPS, and subsequently act as an activator of NF-κB, it has been proposed that this pathway would be characteristic of a Toll-like-receptor-mediated recognition of LPS. The recent use of NOD2 knockout mice has demonstrated that NOD2 does not play an essential role in macrophage response to bacterial products, but rather plays a role in regulating the systemic response to pathogens (129). A number of haplotypes, such as R702W, G908R, and L1007fs, have been linked to the disease. The presence of a susceptibility gene for CD on chromosome 5 has been recently established. When linkage disequilibrium mapping was used, a haplotype on chromosome 5, associated with a cytokine cluster, that confers susceptibility to CD was identified (135). The genotype relative risk for individuals who are heterozygous is twofold compared with approximately sixfold for homozygous individuals.

HLA alleles may also be involved in the genetic predisposition to IBD. For example, an increased frequency of the DR5DQ1 haplotype or the DRB*0301 allele in CD has been described (102). In UC, the HLA DRB1*0103 allele has been associated with extensive disease and extraintestinal manifestations. Other immunoregulatory genes that have been associated with IBD include the intercellular adhesion molecule R241 allele in UC and CD (19) and the IL-1 receptor antagonist allele 2 in UC patients with pancolonic inflammation (175). In summary, all these findings provide evidence that the disease is a genetic disorder. However, other factors, such as an infection, may be needed to trigger the pathological process. Several characteristics of IBD have pointed at acquired factors that may be involved in the etiology of the disease.

Serological Studies

Another approach to study the possible role of MAP in the etiology of CD is to evaluate CD patients for the presence of antibodies reactive against MAP antigens. Antibodies binding to a 38-kDa antigen were demonstrated in 57% of

CD patients, while antibodies to the 24- and 18-kDa bacterioferritin were significantly elevated in 53% of the CD patients (54). However, reaction against those antibodies did not show any correlation with each other, and only 18% of patients were positive for all three antigens. Antibodies to the 45- to -48-kDa antigen were detected in approximately 67% of patients with CD, 10% of patients with UC, and 5% of patients with carcinoma of the colon. Health controls were negative (96). Another MAP antigen against which antibodies have been detected is an antigen of 36 kDa (p36). Patients with CD are usually positive for antip36 antibodies (approximately 89%), while a few UC cases react (approximately 12%). Interestingly, 100% of patients immunized with BCG showed a positive reaction compared with 10% of normal controls (58). Several other studies have demonstrated that CD patients show specific reactivity to the p35 and p36 antigens (116). The predictive value and the sensitivity were 98%, which suggests a strong association between presence of MAP antigens and CD. However, both p35 and p36 are present in MAV, suggesting that a similar reaction will be obtained with the MAV p35 and p36 homologs. A proteomic approach may be used to compare the levels of expression of p35 and p36 in MAP and MAV, with the implication that a specific association of MAP with CD will be underscored by significantly higher levels of expression in MAP. Immune response against the 14-kDa AhpC antigen in a large population of CD patients has also been demonstrated (125). In this case, AhpC expresses constitutively and at high levels in MAP (124).

Other mycobacterial antigens considered are the heat shock proteins. Because of the degree of conservation between bacterial and host 65-kDa proteins, it has been proposed that bacterial antigens might induce antibodies that cross-react with host tissues. As with PCR studies, both positive and negative cross-reactions with serum from CD patients have been reported (15, 152). Likewise, additional studies with antibodies against the 18-kDa bacterioferritin antigen yielded no different results between CD patients and control individuals (168). Negative cross-reactive results against different antigens have also been reported (150).

Other immunological tests useful for the diagnosis of IBD are based on the presence of abnormal IgG and IgA antibodies in IBD patients owing to the development of food allergies (126). Most notably, serum samples from IBD patients develop antineutrophil cytoplasmic antibodies (ANCA) and anti-*Saccharomyces cerevisiae* mannan antibodies (ASCA) (158). Prevalence of ASCA and ANCA antibodies has been found in the range of 50 to 60% in both CD and UC patients (132). A recent report combining ANCA and ASCA markers with the MAP antigens p35 and p36 yielded a test with 95% sensitivity for the detection of CD (143).

Intestinal bacteria have been increasingly implicated as an environmental factor in IBD owing to their mucosal localization and antigenic components. Colonic bacteria are antigenic targets for disease-associated T- and B-cell immune responses in the C3H/HeJBir mouse (18). In this context, Cohavy et al. (41) described the identification of a mycobacterial protein, HupB, for which the presence of specific IgA was associated with CD. It is of relevance that HupB shares cross-reactive epitopes with the mammalian histone H1, the antigen associated with ANCA, a characteristic immune response in IBD. Moreover, MAP and MAV

express more HupB than other mycobacterial species. This observation led to the speculation that HupB-like proteins produced by MAP and other colonic bacteria may trigger the onset of CD. In this way, a MAP-shared antigen (HupB), rather than live bacilli, would be involved in the etiology of CD. It is tempting to speculate that CWD forms of MAP may have a depot effect on the release of HupB and thus contribute to the maintenance of the inflammatory process, at least in some cases of CD.

Chemotherapy

Another approach to identify disease causation is the use of chemotherapeutic agents to eliminate the infectious agents. The caveat of this approach is that antimicrobial agents act against a broad range of bacterial pathogens and may also have a generalized nonspecific effect on the host. In this context, various studies have been or are being conducted to examine the effect of regimens that have been proved active against infections caused by MAV. In one of these trials, CD patients were randomized to receive either clarithromycin (500 mg twice daily) and ethambutol (15 mg/kg/day) or identical appearance placebos for 3 months in addition to their regular therapy. After 3 months of treatment, no signs of improvement were detected in patients with CD (67). Another study used clarithromycin (250 mg twice daily) in combination with rifabutin (150 mg twice daily) in an open clinical trial with 29 patients. In this case, approximately 58% of patients reported significant improvement following the period of therapy. Only five patients were nonresponders (142). Additional large-scale chemotherapeutic trials are in progress.

Animal Models

One of the major problems regarding the establishment of the relationship between MAP and CD is the absence of an animal model that can reproduce the human disease. Animal models have been created to investigate aspects of Johne's disease. Mouse models have been employed to study the MAP response to antibiotics. Some models using immunosuppressed mice, such as the nude mice, have demonstrated multiplication of the bacterium in the intestinal mucosa (71). In addition, formation of the granulomas is in certain cases similar to those observed in either Johne's disease or CD. The SCID-beige mice have also been used with success. Lesions usually develop, compromising the whole intestinal mucosa, following infection with a relatively small number of organisms (113). Another potential model is the Dark Agouti rats because they carry alleles enhancing the susceptibility to autoimmune and inflammatory diseases, including IBD, in mice and humans (91). It would be of interest to determine whether MAP infection of these animals leads to IBD disease that more closely resembles the human condition.

A newborn rabbit model with oral inoculation of bacteria has also been also developed. Nine of 21 rabbits presented granulomatous enteritis, while 14 had di-

arrhea (106). Therefore, although the models can be used to study specific aspects of CD, they are far from ideal to examine if the etiology of CD is due to MAP infection. While infection of sheep is still feasible for the study of Johne's disease, it appears that the goat is the animal in which infection with MAP resembles most lesions of CD (94). Sogawa et al. (145) developed a murine model of granulomatous colitis by intramural injection of mycobacterial cord factor into the intestine. A single dose injected in the form of liposome triggered the appearance of granuloma in the infection site (145). The granuloma was composed of macrophages and CD4 and CD8 lymphocytes. Mucosal erosion or ulceration was not induced. Nonhuman primates have not been investigated. Recent reports have demonstrated that monkeys are excellent models for both tuberculosis and leprosy, as they develop disease that is similar to the human case (27). Although primates are expensive, and their use is under tight regulatory rules, it would be interesting to evaluate if human and bovine strains of MAP would cause intestinal infection that resembles CD. Because the interval between ingestion and development of disease is unknown, this experimental approach is labor intensive, time-consuming, and not without difficulties.

Concluding Remarks

We have presented an overview of the overt or potential pathogenicity of MAP in both human and veterinary medicine. These observations can be summarized as follows. (*a*) Application of the Koch's postulates clearly defined MAP as the etiologic agent of Johne's disease in ruminants, a disease that resembles some aspects of CD in humans. (*b*) Given the close genetic relationship between MAP and MAV and their pathogenicity for a wide range of animal hosts, MAP may be at least an opportunistic pathogen of humans. (*c*) Tropism of MAP for the intestinal mucosa in ruminants suggests a preferential tropism for the gastrointestinal cavity in humans. (*d*) As methods for detection and isolation of MAP in human tissues and serology have improved, the association between CD and MAP colonization has become stronger. This is not the case for UC. (*e*) There are no evident sources of MAP infection for humans, though environmental or wildlife reservoirs have been suggested. Direct transmission from adulterated bovine milk consumption is another less likely possibility.

On the basis of these considerations, the relationship of MAP to CD may be causal, implicating MAP as the etiologic agent of all or a subset of CD cases. Alternatively, the characteristic inflammatory damage of the intestinal mucosa and pattern of cytokine expression in CD patients may create conditions favorable for the colonization of MAP as an opportunistic pathogen. The latter situation may even lead to the maintenance or recrudescence of the inflammatory condition characteristic of CD patients. An etiologic role of MAP in CD would suggest a chemotherapeutic regimen with antimycobacterial agents specific to MAP as the best course of action for the treatment of CD. In contrast, an opportunistic role of MAP would advocate the use of rifabutin or other prophylactic treatment in a fashion similar to that recommended for MAV in AIDS patients. However, if

CD is mediated by an altered immunological reaction to an antigen, including a MAP protein, antimicrobial chemotherapy may be counterproductive. These issues underscore the need to develop suitable animal models of CD to test these divergent hypotheses prior to undertaking extensive clinical trails with antimycobacterial agents. These models may also allow the application of Koch's postulates to CD. Nonetheless, the answer to the role of MAP in CD etiology may come from the consistent results of improved methods of isolation and detection of MAP bacilli or MAP-elicited immune responses in the host, results of animal models, and, eventually, the outcomes of well-designed clinical trials with chemotherapeutic agents. Alternatively, more rapid progress may come from a finding that provides a unified explanation for the different set of phenomena associated with CD. In this context, the analysis of the HupB antigen and other related histone-like proteins may be an important research avenue to pursue that could explain the role of MAP and colonic bacteria in IBD.

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