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On the etiology of Crohn disease

(inflammatory bowel disease/mycobacterial infection/*Mycobacterium paratuberculosis*/*Mycobacterium avium*/cytokines)

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ABSTRACT **Crohn disease (CD) is a chronic, panenteric intestinal inflammatory disease. Its etiology is unknown. Analogous to the tuberculoid and lepromatous forms of leprosy, CD may have two clinical manifestations. One is aggressive and fistulizing (perforating), and the other is contained, indolent, and obstructive (nonperforating) [Gilberts, E. C. A. M., Greenstein, A. J., Katsel, P., Harpaz, N. & Greenstein, R. J. (1994) *Proc. Natl. Acad. Sci. USA* 91, 12721–12724]. The etiology, if infectious, may be due to *Mycobacterium paratuberculosis*. We employed reverse transcription PCR using *M. paratuberculosis* subspecies-specific primers (IS 900) on total RNA from 12 ileal mucosal specimens (CD, $n = 8$; controls, $n = 4$, 2 with ulcerative colitis and 2 with colonic cancer). As a negative control, we used *Myobacterium avium* DNA, originally cultured from the drinking water of a major city in the United States. cDNA sequence analysis shows that all eight cases of Crohn's disease and both samples from the patients with ulcerative colitis contained *M. paratuberculosis* RNA. Additionally, the *M. avium* control has the DNA sequence of *M. paratuberculosis*. We demonstrate the DNA sequence of *M. paratuberculosis* from mucosal specimens from humans with CD. The potable water supply may be a reservoir of infection. Although *M. paratuberculosis* signal in CD has been previously reported, a cause and effect relationship has not been established. In part, this is due to conflicting data from studies with empirical antimycobacterial therapy. We conclude that clinical trials with anti-*M. paratuberculosis* therapy are indicated in patients with CD who have been stratified into the aggressive (perforating) and contained (nonperforating) forms.**

Crohn disease (CD) is a chronic, panenteric inflammatory disease of the gastrointestinal tract (1). Its etiology is unknown; however, several possibilities have been suggested. CD may be a primary autoimmune disease due to the pathogenic effect of cytokines (2). Intestinal vasculitis has also been implicated (3). Proposed infectious causes include persistent measles virus (4) and mycobacteria, particularly *Mycobacterium paratuberculosis* (5–9).

Only the lack of identifiable acid/alcohol fast bacilli on Ziehl–Neelsen staining on histopathological specimens differentiates CD from intestinal tuberculosis. Thus, the thesis that CD may be due to a mycobacterial infection is appealing. However, failure of well-performed clinical trials with empirical antimycobacterial therapy (10–17) has led many to conclude that CD cannot be ascribed to a mycobacterial infection (14–17).

Although controversial (18, 19) there is clinical (20, 21), epidemiological (22), and molecular (23, 24) evidence indicating that there are two distinct clinical manifestations of CD. These CD subclassifications have been designated as "perforating" and "nonperforating" forms (22). Patients with perforating CD have abscesses and/or free perforation. Perforating CD is the more aggressive form, with a higher reoperation rate (21, 22). In contrast, nonperforating CD has a more indolent clinical course and is associated with obstruction and bleeding (22).

This dual clinical presentation renders CD analogous to other mycobacterial diseases such as leprosy, which has two clinical manifestations, the contained tuberculoid and the uncontrolled lepromatous forms (25). Another mycobacterial infection with a dual presentation in humans is tuberculosis, which has the contained (Ghon focus or pulmonary apical scarring) form rather than the aggressive (miliary) form.

We hypothesized that CD may indeed be due to a mycobacterial infection, possibly from *M. paratuberculosis*. We speculate that the inability to document a response to empirical antimycobacterial therapy (14–17) of CD might be ascribable to the failure to separate the CD patients into perforating and nonperforating clinical forms before study.

We have had the opportunity to evaluate excised ileal mucosal tissue from the perforating and nonperforating CD subpopulations as well as four control patients. Samples from this cohort had previously been used in a study that demonstrated a difference in the cytokine profiles between the two CD subpopulations (24). In this study, we employ reverse transcription (RT)-PCR amplification to probe for the presence of a *M. paratuberculosis*-specific DNA sequence (IS 900) (26).

METHODS

We assigned patients to control, perforating CD, or nonperforating CD ($n = 4$ for each group), following previously reported preoperative clinical and radiological findings (22). The diagnosis, demographic data, disease duration, and histopathological features of this patient population have been presented in detail (24). All patients were having clinically indicated intestinal resections. Patients with CD had active disease at the time of surgery. Control patients were having resections for right-sided colonic cancer or closure of an ileostomy after prior resection for histologically proven ulcerative colitis (24). All samples used were from a cytokine study (24) that had been approved by the Institutional Review Board at the Veterans Affairs Medical Center (Bronx, NY; IRB 0720-14 1993) and at the Mt. Sinai Medical Center (New York, NY; GCO 93-514 SUX).

All studies were performed on ileal mucosal samples. Two immediately adjacent areas from the region of maximal pathology (≈2 g each) were excised from the resected bowel segment. Perforating specimens were immediately adjacent to a fistula orifice and nonperforating specimens were from within a stricture. One specimen was used for histopathological analysis using routine hematoxylin/eosin stains (24). For molec-

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Abbreviations: CD, Crohn disease; RT, reverse transcription; IL-1β and IL-6, interleukins 1β and 6.

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ular analysis, total RNA was prepared as described (24) and stored in water treated with diethylpyrocarbonate (Sigma) at −80°C until further processed. RT in this study differed from that previously reported (24) only in that random primers (GIBCO/BRL) rather than oligo dT primers (Invitrogen) were used.

PCR primers were commercially purchased (Brookdale Center for Molecular Biology, Mt. Sinai School of Medicine, New York). The RNAs amplified include a housekeeping gene (β-actin; ref. 25), the cytokines interleukin 1β (IL-1β) and IL-6 (27), *M. paratuberculosis*-specific sequences (IS 900; ref. 28), and *Myobacterium avium* subsp. *silvaticum*-specific sequences (IS 902; ref. 29). PCR conditions were as described (24). Annealing temperatures were individualized for each set of primers (Table 1). The *M. paratuberculosis* assay was performed five times.

M. paratuberculosis (ref. 31; ATCC 43545) and *M. avium* (a gift of T. Aronson; ref. 32) were used as controls. To obtain DNA for use as the PCR template, a minor modification of a plasmid purification system was used (Wizard; Promega). An aliquot of the culture specimen was extracted in boiling water for 10 min. After centrifugation, the resulting pellet was resuspended in 1 ml of lysozyme (10 mg/ml, Sigma) and incubated at 37°C for 20 min before processing as directed by the manufacturer (Promega).

After PAGE (6% polyacrylamide Gel; 10 μl of PCR product with 2 μl of loading buffer; ref. 33), the dried gel was autoradiographed (Kodak XAR-2) for 30 min to 1 week. PCR bands that comigrated with the control *M. paratuberculosis* (31) were excised, reamplified (Vent DNA polymerase; NEB, Beverly, MA) and subcloned (Primer PCR Cloner Cloning System; 5 Prime – 3 Prime, Inc.). The resulting plasmid was transformed, amplified, purified (Wizard Minipreps; Promega) and repurified (Centricon 100; Amicon). After restriction digestion (*Bam*HI; NEB, Beverly, MA), the resulting band (≈350 bp; see Fig. 1) was DNA sequenced commercially (Brookdale Center of Molecular Biology) using an Automated Bioscience Sequencer. Sequence comparisons were performed using MacVector (Kodak–BRL).

RESULTS

Data from the RT-PCR amplification of total RNA are presented as autoradiographs (Fig. 1). There is cDNA synthesis in all samples during RT. The signal from the control housekeeping gene, β-actin (Fig. 1), indicates insignificant degradation of the total RNA, which had been stored at −80°C in water treated with diethylpyrocarbonate for more than 2 years. All of the patients with inflammatory bowel disease have a band that comigrates with the *M. paratuberculosis* control (Fig. 1, fourth row). The *M. paratuberculosis* data were replicated on five separate occasions. There is no signal in the patient samples when evaluated with *M. avium* subsp. *silvaticum*-specific primers (Fig. 1, fifth row). The results obtained using IL-1β and IL-6 specific primers (Table 1) have the same pattern as that in the original study (24). These data indicate that the *M. paratuberculosis* results cannot be attributed to intersample contamination.

The sequences of the subcloned bands (Fig. 1) were compared with the GenBank sequence (IS 900) of *M. paratuberculosis*. Sequence from patients have 95–98.6% homology with the GenBank sequence of IS 900 *M. paratuberculosis* sequence (Fig. 2). These data indicate that the signal detected in the CD and ulcerative colitis patients are indeed ascribable to the presence of *M. paratuberculosis* DNA in those 10 specimens.

In our experiments, as a positive control, we used a sample of *M. avium* (sample 51; ref. 32). This sample gave a positive result with *M. paratuberculosis*-specific (IS 900) primers. It gave appropriate positive signal with two nonspecific mycobacterial 16S ribosomal primers (Fig. 3; ref. 30) but no signal with *M. avium* subsp. *silvaticum*-specific (IS 902) primers. Accordingly, this band from *M. avium* was subcloned and sequenced. DNA sequence comparisons with the GenBank sequence of *M. paratuberculosis* (ref. 31; Fig. 2) show that sample of *M. avium* (51; ref. 32) is in fact the *M. avium* subspecies *M. paratuberculosis*.

DISCUSSION

The etiology of CD remains an enigma. It would appear that three factors are necessary for CD to become manifest in any given individual patient. First, there should be a precipitating ligand or "trigger." Second, this ligand must be transmitted to an individual host. Finally, the host must be susceptible to developing CD when exposed to the ligand. In this manuscript, we have addressed the first two of the three prerequisites necessary for the development of clinical CD.

Others have shown by RT-PCR (34, 35) or RT-PCR followed by Southern blotting (36) that *M. paratuberculosis* is indeed found in the intestine of patients with CD and ulcerative colitis as well as in samples from some normal controls (36). Indeed, some authors have concluded that, because of its high prevalence, *M. paratuberculosis* cannot have an etiological role in the pathogenesis of CD (36). We demonstrate here by sequence analysis that the bands that comigrate with *M. paratuberculosis* from patients with CD actually have the DNA sequence of *M. paratuberculosis*.

Table 1. Technical specification of PCR primers used in this study

Primer	Ref.	Annealing temperature, °C	No. of base pairs	Sequence
β-Actin up	25	55	20	5′-GTGGGGCGCCCCAGGCACCA-3′
β-Actin down	25	55	24	5′-CTCCTTAATGTCACGCACGATTTC-3′
IL-1β up	27	55	26	5′-AAACAGATGAAGTGCTCCTTCCAGG-3′
IL-1β down	27	55	24	5′-CTCCTTAATGTCACGCACGATTTC-3′
IL-6 up	27	55	20	5′-CCTTCTCCACAAGCGCCTTC-3′
IL-6 down	27	55	21	5′-GGCAAGTCTCCTCATTGAATC-3′
IS 900 up	28	58	25	5′-TGGACAATGACGGTTACGGAGGTGG-3′
IS 900 down	28	58	25	5′-TGATCGCAGCGTCTTTGGCGTCGGT-3′
IS 902 up	29	58	18	5′-CTGATTGAGATCTGACGC-3′
IS 902 down	29	58	19	5′-TTAGCAATCCGGCCGCCCT-3′
16 S 285 up	30	58	21	5′-GAGAGTTTGATCCTGGCTCAG-3′
16 S 264 down	30	58	20	5′-TGCACACAGGCCACAAGGGA-3′
16 S 248 up	30	58	20	5′-GTGTGGGTTTCCTTCCTTGG-3′
16 S 261 down	30	58	20	5′-AAGGAGGTGATCCAGCCGCA-3′

Primer sequence, length, and source of sequence are as indicated. Primers have been previously published (25, 27–30). Annealing temperature was individualized for each set of primers. Other parameters are as published (24). up, Upstream primer; down, downstream primer.

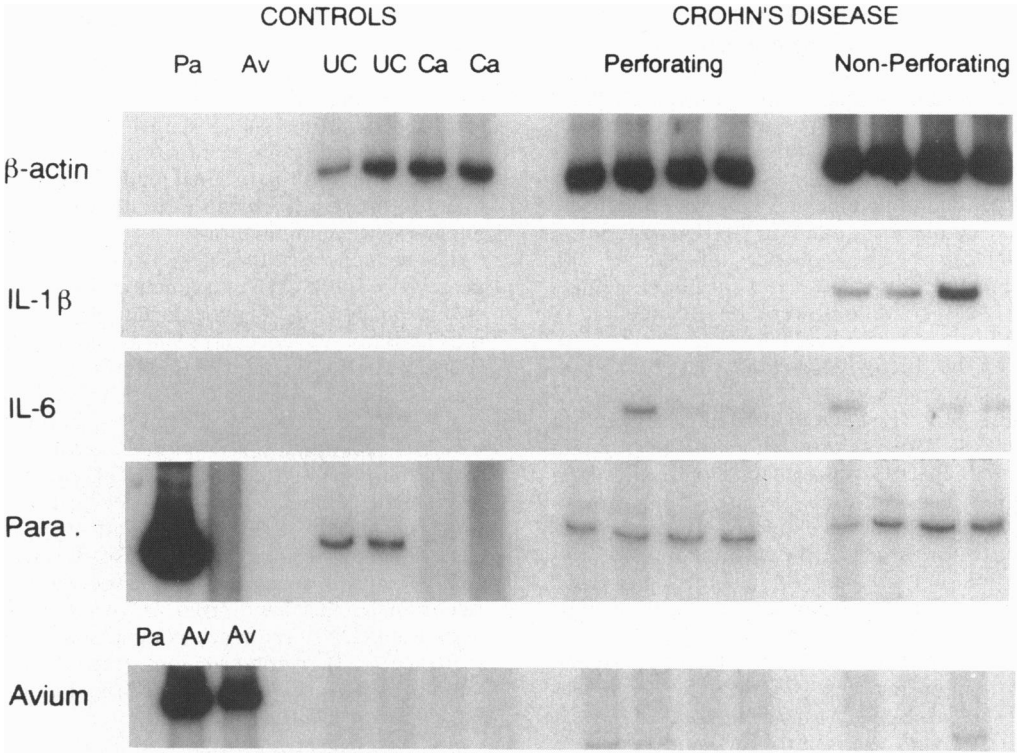

FIG. 1. Representative autoradiographs of PAGE. The mRNA being evaluated is shown on the left, and clinical divisions are indicated at the top. Density of signal depends on the length of exposure. β-Actin signal is present in all samples, indicating that the RNA has, at worst, been incompletely degraded during 2 years of storage. Para. and Pa, *M. paratuberculosis* (31); Avium and Av, *M. avium* subsp. *silvaticum* (32); Ca, patients with colonic cancer; UC, patients with ulcerative colitis. The patterns presented are identical to that seen in these samples when originally assayed (24), indicating that there has been no contamination during laboratory manipulation. *M. paratuberculosis* signal is seen in all 10 patients with inflammatory bowel disease (fourth row). There is no signal seen in patient samples when the primers used are for the *M. avium* subsp. *silvaticum* (fifth row).

RT-PCR is an exquisitely sensitive technique. Thus, the possibility of intersample contamination resulting in erroneous data must always be considered. In this study, we used the same patient samples that were used and evaluated previously (24). In the first study (24), we demonstrated that IL-1β differs between the two forms of CD. Additionally, IL-6 is not found in the control specimens (24). In this study, we replicated the different IL-1β and IL-6 patterns (24). Thus, the present *M. paratuberculosis* results cannot be ascribed to intersample contamination.

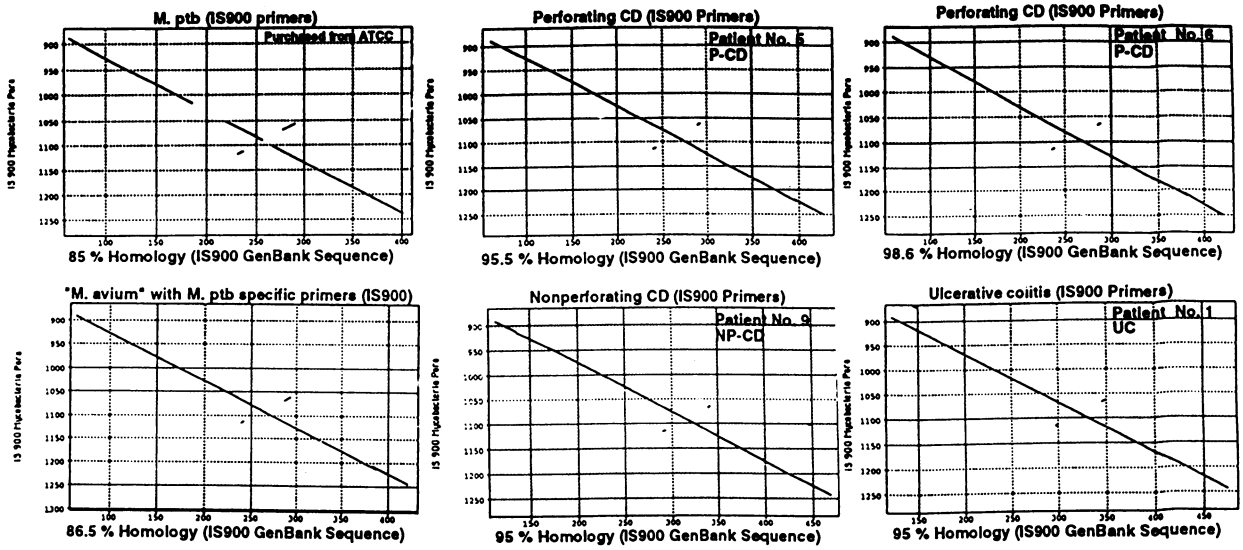

FIG. 2. Graphic representation of the sequence homology of the subcloned fragment generated using the *M. paratuberculosis*-specific primers (IS 900; ref. 28). Sequence homology was calculated using MacVector (Kodak–BRL). Results are expressed as a % homology with the GenBank sequence for *M. paratuberculosis* (31). The *M. avium* control is in fact *M. paratuberculosis*. Comparisons of % homology with the GenBank sequence for *M. paratuberculosis* are presented graphically for four representative patient samples (three from patients with CD and one from a patient with ulcerative colitis, for the ATCC *M. paratuberculosis* and sample *M. avium* 51). These data indicate that all samples are *M. paratuberculosis*. Similar data were obtained but are not presented for other CD and ulcerative colitis patients.

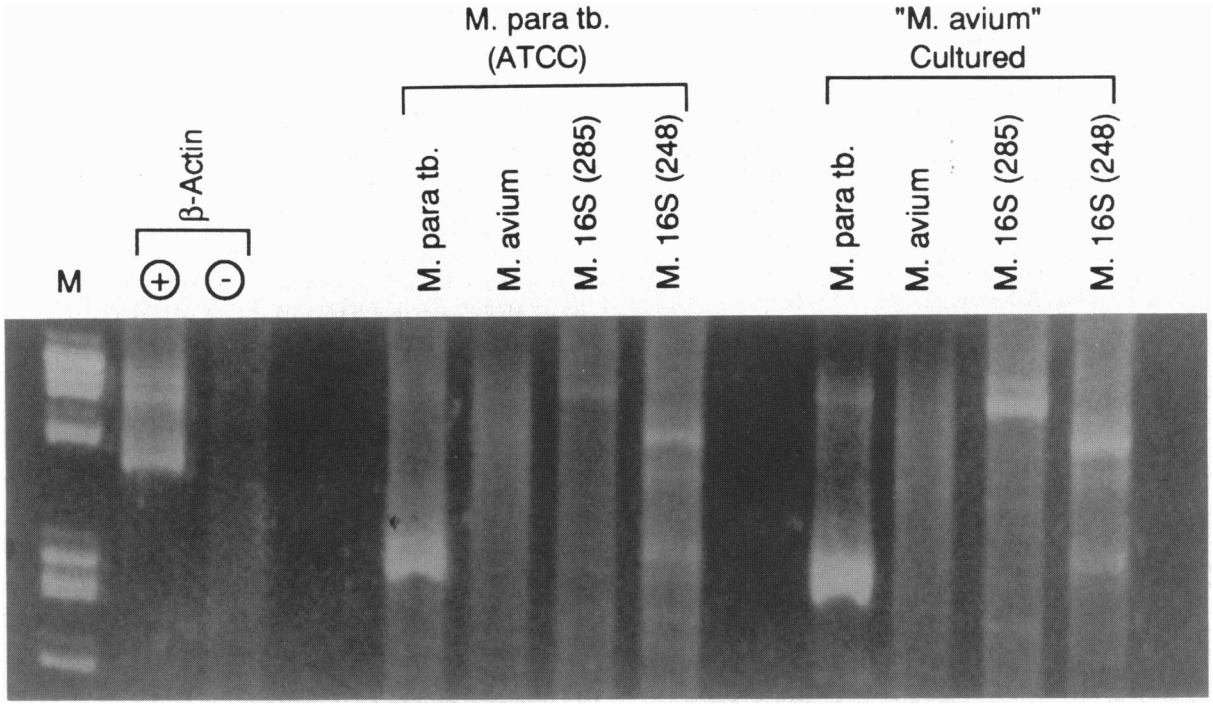

FIG. 3. Comparison of *M. paratuberculosis* (31) and "*M. avium*" (sample 51; ref. 32). Primers pairs were as follows: M. para tb, *M. paratuberculosis*-specific (IS 900; Table 1; ref. 28); M. avium, *M. avium* subsp. *silvaticum* (IS 902: Table 1; ref. 29) and nonspecific mycobacterial ribosomal mycobacterial 16S (285; Table 1; ref. 30) and mycobacterial 16S (248; Table 1; ref. 30). This IS 900 band is from *M. avium* and was subsequently sequence analyzed (see text and Fig. 2). These data indicate that *M. avium* sample 51 is in fact *M. paratuberculosis*.

Because of the similarity of CD to intestinal tuberculosis, the thesis that CD should be ascribed to *M. paratuberculosis* is very appealing. However, there has been a failure to change the clinical course of CD with empirical antimycobacterial therapy (10–17). We suggest a possible explanation for this inability to show a *M. paratuberculosis* cause and effect in CD. It is that CD has been, in our opinion erroneously, conceptualized as a homogenous disease.

Mycobacterial diseases may have widely divergent clinical presentations. Tuberculosis, in its contained manifestation, may merely have a positive purified protein derivative skin test reaction, a Ghon focus, or simple pulmonary apical scarring. In contrast, in an immune compromised individual tuberculosis may present as the aggressive miliary form. Similarly, leprosy is stratified into the contained tuberculoid or the aggressive proliferative lepromatous forms (25). We suggest that if patients manifesting the polar manifestations of either tuberculosis or leprosy were grouped together, controlled trials of treatment with effective agents would result in disappointing or uninterpretable results.

Analogous to tuberculosis and leprosy, we suggest that CD has two clinical presentations. They are the aggressive (perforating) and contained (nonperforating) forms (21–24). The total number of patients evaluated, which supports this division, is 891 (20, 21, 23, 24), compared with 349 patients in whom such a separation is not demonstrated (18, 19). Accordingly, we now suggest that CD should similarly be stratified into the perforating and nonperforating subdivisions before evaluating any therapeutic intervention in CD (37).

Table 2. Suggested comparisons between acknowledged mycobacterial diseases and CD

Diseases	Clinical manifestation	
	Indolent/contained	Aggressive
Tuberculosis	Positive purified protein derivative/Ghon focus, lung apical scarring	Miliary tuberculosis
Leprosy	Tuberculoid	Lepromatous
CD	Nonperforating (obstructive)	Perforating (fistulizing/ abscesses)

Additionally, the use of suboptimal anti-*M. paratuberculosis.* therapy may have resulted in an inability to detect an appropriate therapeutic response (38). Thus, we hypothesize that *M. paratuberculosis* may indeed be pathogenic in CD. To test this hypothesis, it will be necessary to perform studies on patients with CD using the appropriate antimycobacterial therapy for *M. paratuberculosis*. At present, the recommended drugs are clarithromycin (38) and ethambutol. However, it will be necessary to subdivide CD patients into the contained nonperforating and the aggressive perforating forms before initiating appropriate studies.

Koch's postulates have not, to this day, been met for leprosy (39). It is possible that even if *M. paratuberculosis* is indeed the etiological agent in CD, Kochs' postulates will not be satisfied. Nevertheless, we hypothesize that it will be the aggressive (perforating/fistulizing) form that will best respond to the appropriate anti-*M. paratuberculosis* therapy (38). In contrast, the nonperforating form may be analogous to pulmonary apical scarring seen in burnt-out pulmonary tuberculosis or tuberculoid leprosy. The nonperforating form of CD is consequent to a heightened proinflammatory cytokine response (24). Therefore, we also hypothesize that, although controversial (37), nonperforating CD is more likely to respond to conventional antiinflammatory therapies such as corticosteroids and immunosuppression (40–42).

During the course of our experiments, we showed by both PCR primer analysis as well as DNA sequence analysis that a mycobacterium, simply labelled as *M. avium* is in fact the *M. avium* subspecies *M. paratuberculosis*. This specimen had originally been cultured from the municipal water supply of a major city in the United States. These data imply that *M. paratuberculosis* may well be as ubiquitous in the environment

as *M. avium* (32). We speculate that CD may be the consequence when *M. paratuberculosis* is exposed to a susceptible individual.

This study does not address why some individuals will remain asymptomatic while others will progress to clinical CD; nor does it address the possible implications of the *M. paratuberculosis* signal noted in the specimens from individuals with ulcerative colitis.

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