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Results of Multiple Diagnostic Tests for *Mycobacterium avium* subsp. *paratuberculosis* in Patients with Inflammatory Bowel Disease and in Controls

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Mycobacterium avium* subsp. *paratuberculosis* has been incriminated as a cause of Crohn's disease (CD); however, studies to date have been relatively small and generally only used a single diagnostic assay. The objective of the study was to reexamine the association of *M. avium* subsp. *paratuberculosis* and CD using multiple diagnostic tests. Five methods were used to detect *M. avium* subsp. *paratuberculosis* infections in 439 inflammatory bowel disease (IBD) patients and 324 control subjects in the United States and Denmark. Most assays were adaptations of diagnostic tests for this infection performed routinely on animals. PCR for IS*900*, a genetic element unique to *M. avium* subsp. *paratuberculosis*, was positive significantly more often on resected bowel and lymph node tissues from CD patients (19.0%) and ulcerative colitis (UC) patients (26.2%) than from controls (6.3%) ($P < 0.05$). Positive IS*900* PCR results occurred more often in U.S. than in Danish IBD patients, 32.0 versus 13.3% ($P = 0.025$). The majority of Danish patients were bacillus Calmette-Guérin (*Mycobacterium bovis* BCG) vaccinated (CD, 77.5%; UC, 86.6%; controls, 83.0%) whereas none of the U.S. patients with IBD and only 2% of U.S. controls were vaccinated. Among Danish IBD patients, positive PCR findings were four times more common among subjects who were not BCG vaccinated (33.3%) than among BCG vaccinates (8.8%, $P = 0.02$). Culture of the same tissues tested by PCR using modified BACTEC 12B medium failed to grow *M. avium* subsp. *paratuberculosis* from patients or controls. U.S. CD patients had the highest serological evidence (enzyme-linked immunosorbent assay [ELISA] for serum antibodies) of *M. avium* subsp. *paratuberculosis* infection (20.7% of patients positive) which was higher than for all UC patients studied (6.1%) or healthy controls (3.8%, $P < 0.005$). Among Danish patients alone, however, no significant differences in rates of ELISA-positive results among CD, UC, or control patients were found. For 181 study subjects, both IS*900* PCR and ELISA were performed. Although 11 were ELISA positive and 36 were PCR positive, in no instance was a patient positive by both tests, suggesting that these states are mutually exclusive. Evaluation of cytokine-mediated immune responses of IBD patients was complicated by the influence of immunosuppressive therapy given most IBD patients. Gamma interferon (IFN-γ) release by peripheral blood leukocytes after *M. avium* purified protein derivative PPD antigen stimulation showed significantly lower responses in CD patients than in UC patients or controls in both U.S. (by ex vivo assay) and Danish (by in vitro assay) populations ($P < 0.05$). Interleukin-5 responses were not different among CD, UC, or control groups. Collectively, the PCR, ELISA, and IFN-γ tests for *M. avium* subsp. *paratuberculosis* together with the unexpected observation that BCG vaccination influenced *M. avium* subsp. *paratuberculosis* detection, lead us to conclude that *M. avium* subsp. *paratuberculosis*, or some similarly fastidious mycobacterial species, infects at least a subset of IBD patients. Whether the infection is primary (causal) or secondary, it may contribute to the etiopathogenesis of IBD.

Mycobacterium avium subsp. *paratuberculosis*, formerly called *Mycobacterium paratuberculosis* (68) causes a chronic granulomatous ileitis in ruminant animals, known as Johne's disease (13, 33, 66). Given the similarity of clinical signs (diarrhea and weight loss), pathology (transmural diffuse granulomatous inflamation), and epidemiology (rising incidence, long incubation period, and familial occurrence pattern) between Johne's disease and Crohn's disease (CD), coupled with positive diagnostic tests for *M. avium* subsp. *paratuberculosis*, some investigators have proposed that *M. avium* subsp. *paratuberculosis* is the etiologic agent of CD (9–11, 18, 27, 31, 32, 41, 45, 49, 50, 52, 59, 72, 75). Controversy about this theory continues, in part, due to conflicting evidence and differences in laboratory techniques among studies (8, 67, 71). In addition, most prior studies examined relatively small numbers of patients, and the testing laboratories often were not blinded to patient disease status, causing skeptics to question the validity of the data. The purpose of the study herein described was to apply multiple diagnostic tests for paratuberculosis, those proven to be most accurate for diagnosis of paratuberculosis in animals, to larger populations of inflammatory bowel disease IBD patients and controls to clarify if *M. avium* subsp. *paratuberculosis* is a zoonotic agent involved in the pathogenesis of IBD.

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TABLE 1. Characteristics of study groups in the United States and Denmark[a]

Characteristic	CD				UC	
	Surgical		Outpatient		Surgical	
	US	DK	US	DK	US	DK
Total no.	33	50	114	75	35	26
Gender; no. (%) of males	15 (45.5)	17 (34)	46 (40.4)	35 (47)	19 (54.3)	16 (62)
Age (yr), mean ± SD	34.2 ± 13.6	39.4 ± 14.0	40.9 ± 13.4	44.8 ± 17.6	38.4 ± 12.4	42.5 ± 14.7
No. BCG vaccinated (%)	0	37 (78.7)	0	56 (88.9)	0	20 (80.0)
Disease duration (yr), mean ± SD (range)	9.5 ± 8.7 (1–37)	7.8 ± 7.32 (0–26)	13.3 ± 11.7 (0–40)	9.1 ± 9.3 (0–41)	6.5 ± 6.3 (0–23)	7.0 ± 8.0 (0–25)

[a] Abbreviations: US, United States; DK, Denmark; NR, not reported; NA, not applicable. For percent BCG vaccinated, subjects with unknown vaccination status were excluded from the calculation.

MATERIALS AND METHODS

Patients and controls. Roughly equal numbers of patients with CD ($n = 272$), patients with ulcerative colitis (UC; $n = 167$), and controls ($n = 275$) originated from Wisconsin and Copenhagen County, Denmark. Both outpatients and patients who underwent surgery for their disease with resection of small and/or large bowel segments were included in the study. The number and clinical characteristics of patients in each study group are given in Table 1. Disease diagnosis was confirmed according to internationally accepted criteria (4). The study protocol was approved by the appropriate human subject committees. All patients and healthy controls signed approved consent forms.

***Mycobacterium bovis* BCG vaccination status of study subjects.** Information on treatment history for IBD and tuberculosis (BCG) vaccination status was obtained from all patients and controls, as these factors had the potential to influence diagnostic test results for mycobacterial infection. Prior to 17 December 1986, BCG vaccination for school-age children in Denmark was routine (54). After that date, it was restricted to Mantoux test-negative children more than 18 years old belonging to a high-risk group for tuberculosis. Thus, in general, Danish study subjects over 18 years old at the time that most samples were collected usually were BCG vaccinated.

Culture for *M. avium* subsp. *paratuberculosis*. Surgical patients provided resected bowel and lymph node tissues. In the attempt to detect *M. avium* subsp. *paratuberculosis* by culture, modified radiometric (BACTEC) 12B broth culture media were used. Tissues were collected at the time of surgery from CD, UC, and control patients. They were transported directly to the laboratory with refrigeration, arriving within a maximum of 4 h. The BACTEC method for isolation of *M. avium* subsp. *paratuberculosis* has been described previously (16) and independently corroborated as a sensitive technique for *M. avium* subsp. *paratuberculosis* detection (78). Six sites in each resected bowel specimen were sampled as follows: site 1, proximal end of resection—usually normal-appearing bowel; site 2, proximal end of pathologic bowel; site 3, upper middle section of pathologic bowel; site 4, lower middle section of pathologic bowel; site 5, distal edge of pathologic bowel; and site 6, distal end of the resection—usually normal-appearing bowel. Each sample weighed 0.7 g on average.

Intestinal tissues were surface disinfected by agitation in 1.0% hexadecylpyridinium chloride (HPC) for 15 min. After rinsing the tissue in sterile saline to remove the HPC, they were homogenized for 4 min in sterile isotonic saline (1:5 tissue-to-saline ratio) in a sterile plastic bag using a Stomacher (Seward Medical Ltd., London, United Kingdom). Up to four lymph nodes per patient were collected aseptically and individually homogenized without HPC decontamination. Each homogenate (0.1 ml only for Danish patients and 0.1 ml for one set of cultures and 0.5 ml for a second identical set of cultures for U.S. patients) was then transferred to three BACTEC 12B vials. The remaining homogenate was used for IS*900* PCR analysis. All BACTEC vials were supplemented with 1.0 ml of egg yolk suspension (Difco Laboratories, Detroit, Mich.) and Mycobactin J (Allied Monitor Inc., Fayette, Mo.). One of the three vials contained no antibiotics. The second contained vancomycin (10 μg/ml), amphotericin B (20 μg/ml), and nalidixic acid (30 μg/ml). The third contained only amphotericin B and nalidixic acid. Different antibiotic combinations were used for trying to limit culture contamination while simultaneously using as little of the antibiotics as possible to avoid unwanted inhibition of *M. avium* subsp. *paratuberculosis* growth. Thus, up to 10 total tissues per patient were placed in culture with three cultures per tissue homogenate set up in Denmark and six cultures per tissue homogenate set up in the United States. Positive and negative control tissues from animals were included regularly. Cultures were incubated for 3 years before being discarded.

When the growth index reading was above 30 in any BACTEC vial, 0.1 ml of vial contents was plated on blood agar and a smear was stained by both Gram's and acid-fast methods and examined by light microscopy. Contaminants were characterized only by colonial and cellular morphology and Gram stain reaction. Mycobacterial isolates other than *M. avium* subsp. *paratuberculosis* were identified by standard methods at mycobacterium reference laboratories.

Detection of *M. avium* subsp. *paratuberculosis* DNA by IS*900*-specific PCR on tissues. (i) DNA extraction. The tissue (100 to 300 mg) was incubated on an IKA Vibrax VXR vibrator (Janke & Kunkel, Staufen, Germany) for 18 h at 35°C in 2.5 ml of 100 mM NaCl–100 mM EDTA (pH 8.0)–0.25% Tween 20–0.5% sodium dodecyl sulfate–0.5 g of proteinase K (Sigma, St. Louis, Mo.) per liter. Subsequently, NaOH was added to 0.2 M and incubated for 20 min at 100°C. Proteins were precipitated by addition of 0.7 M sodium acetate (pH 7.0) and $CHCl_3$-isoamyl alcohol (24/1 ratio). The water-top phase was removed and extracted once more if not clear; otherwise the DNA was precipitated by addition of 2-propanol. The precipitate was resuspended in TE (10 mM Tris, 1 mM EDTA; pH 8.0) and reprecipitated once by the addition of sodium acetate and absolute ethanol. The DNA pellet was washed in 70% ethanol, air dried, and resuspended in TE. DNA concentration and purity were evaluated by UV absorption spectrophotometry.

(ii) DNA amplification. IS*900* PCR was conducted as a nested-primer PCR as previously described (41) with minor modifications, which included incorporation of dUTP in PCR amplicons as carryover prevention (44) and the inclusion of 0.01 mM digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) in the inner PCR for detection purposes. In addition to carryover prevention, extensive precautions were taken to avoid contamination as described by Kwok and Higuchi (39).

(iii) Detection. The PCR products in the inner PCR were analyzed by standard agarose gel electrophoresis. Furthermore, the PCR products were also visualized by enzyme-linked immunosorbent assay ELISA detection. Five microliters of PCR product from the inner PCR was incubated at 95°C for 10 min in 20 mM sodium phosphate (pH 6.5) containing an IS*900*-specific biotin-PNA probe [peptide nucleic acid: biotin-(H)-TCGCTCCTCGA-(NH_2); PNA Diagnostics, Copenhagen, Denmark]. Following cooling on ice, 200 μl was transferred to a streptavidin-coated microtiter well (Boehringer Mannheim) and incubated for 2 h at 37°C. The PCR products were detected by peroxidase-coupled antidigoxigenin Fab fragments (Boehringer Mannheim) according to the manufacturer's instructions. Positive and negative control tissues from animals were included and tested blind.

The tissue (each sample of 100 to 300 mg) was incubated on an IKA Vibrax VXR vibrator at 1,200 rpm for 18 h at 35°C in 2.5 ml of 100 mM NaCl–100 mM EDTA (pH 8.0)–0.25% Tween 20–0.5% sodium dodecyl sulfate–0.5 g of proteinase K (Sigma) per liter. Subsequently, NaOH was added to achieve 0.2 M and incubated for 20 min at 100°C. Proteins were precipitated by addition of 2 ml of 0.7 M sodium acetate (pH 7.0) plus 4 ml of $CHCl_3$-isoamyl alcohol (24:1), vortexing for 15 s, incubating at room temperature for 15 min, and centrifuging at 5,000 × *g* for 20 min. The water-top phase was removed and extracted once more if not clear; otherwise the DNA was precipitated by addition of 3 ml of 2-propanol, overnight incubation at −20°C, and centrifugation at 6,200 × *g* for 30 min. The precipitate was resuspended in 200 μl of Tris (10 mM)–EDTA (1 mM), pH 8.0, and reprecipitated once by the addition of 40 μl of 4 M sodium acetate (pH 7.0) plus 1,200 μl of absolute ethanol, incubation overnight at 4°C, and centrifugation at 13,000 × *g* for 30 min. The DNA pellet was washed in 70% ethanol, air dried, and resuspended in 200 μl of Tris (10 mM)–EDTA (1 mM), pH 8.0. DNA concentration and purity were evaluated by UV absorption spectrophotometry at 260 and 280 nm (A_{260}/A_{280}).

ELISA for serum antibodies to *M. avium* subsp. *paratuberculosis*. The ELISA for serum antibodies to *M. avium* subsp. *paratuberculosis* in cattle (*M. paratuberculosis* antibody test kit; IDEXX Laboratories, Inc., Westbrook, Maine) was adapted for human use. *M. avium* subsp. *paratuberculosis* antigen-coated 96-well microtiter plates and *Mycobacterium phlei*-containing serum diluent (used to absorb cross-reactive antibodies) supplied with the kit were used. The kit employs a horseradish peroxidase-labeled protein G conjugate to detect binding of antibodies to the solid-phase *M. avium* subsp. *paratuberculosis* antigens. On each ELISA plate, positive and negative bovine and human control sera were tested in duplicate along with bovine control sera provided with the kit. As a positive control human serum, we used blood donated by a Wisconsin veterinarian who

TABLE 1—*Continued*

UC		Control			
Outpatient		Surgical		Healthy	
US	DK	US	DK	US	DK
31	75	19	30	198	77
10 (29.4)	41 (55)	11 (57.9)	16 (53)	84 (42.4)	40 (52)
44.2 ± 12.4	48.3 ± 16.4	43.8 ± 20.1	67.8 ± 15.6	37.8 ± 13.5	42.6 ± 12.1
0	67 (88.9)	0	20 (76.9)	4 (2.0)	63 (85.1)
11.5 ± 10.1 (0–36)	12.4 ± 12.0 (0–53)	NR	0.2 ± 0.9 (0–4)	NA	NA

was accidentally inoculated with the commercial killed vaccine for Johne's disease, resulting in a persistent localized granulomatous tissue reaction at the injection site. A commercial pool of serum from normal healthy blood donors (Binding Site, Birmingham, United Kingdom) was used as the negative human control.

Study subject sera were diluted 1:20 in kit serum diluent (identical to the kit protocol for testing bovine sera) and incubated at room temperature for 30 min prior to transfer to the microtiter plate. One hundred microliters of each diluted serum sample and control serum was dispensed into duplicate microtiter wells and incubated for 30 min at room temperature. Unbound serum components were then removed by washing wells six times with phosphate buffer provided with the kit. After removal of residual wash fluid, 100 μl of conjugate was added and the plate was incubated another 30 min. After washing the wells as before, 100 μl of TMB substrate solution was added to each well and incubated for 15 min. The enzymatic reaction was stopped after 15 min by addition of 100 μl of stop solution provided with the kit. The optical density (OD) for each well was measured at 650 nm (BioTek EL312; BioTek Instruments, Inc. Winooski, Vt.). Assays were considered valid based on kit manufacturer's interpretation guidelines.

Mean ELISA OD readings for patient sera were transformed and expressed as the sample-to-positive (s/p) ratio as done for the cattle assay; however, OD values for the human sera were used as controls in the calculation s/p = OD of sample − OD of negative control/OD of positive control − OD of negative control. Results of testing 252 sera from Red Cross (Madison, Wis.) blood donors using this prototype ELISA for human serum antibodies to *M. avium* subsp. *paratuberculosis* were used to establish the s/p cutoff for a positive test.

IFN-γ assay on whole blood (ex vivo assay) performed on U.S. study subjects. Venous blood was collected in heparinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, N.J.). Blood was always collected prior to or at surgery for subjects undergoing bowel resections. Within 4 h, 1.0 ml was aliquoted into each of four tubes. The first tube received no antigens or mitogens and served as the baseline circulating gamma interferon IFN-γ control. To the second tube was added 45 μg of *M. bovis* purified protein derivative (PPD). To the third tube was added 45 μg of *M. avium* PPD. To the fourth tube was added 5 μg of phytohemagglutinin (PHA; Murex Diagnostics Ltd., Dartford, England) as a positive control for viability and IFN-γ production capacity of the patient's leukocytes. All tubes were incubated for 48 h at 37°C. Plasma was harvested from each of the four tubes and assayed for IFN-γ using a commercial ELISA kit for human IFN-γ (Quantikine; R & D Systems, Minneapolis, Minn.). This sandwich ELISA yields OD units that are linear with the log concentration of human IFN-γ concentration. A standard curve was performed with each assay using kit controls.

Preliminary studies were done on the positive-control individual accidentally inoculated with *M. avium* subsp. *paratuberculosis* vaccine. This person responded to both *M. bovis* and *M. avium* PPD by release of >600 pg/ml of plasma. Leukocytes from multiple healthy controls showed negligible IFN-γ responses to both PPD preparations. A comparable ex vivo whole blood assay has been used for diagnosis of paratuberculosis in cattle (58, 79), cervical lymphadenitis caused by *M. avium* in children (17, 36), and leprosy (76, 77).

IFN-γ and interleukin-5 IL-5 release by PBMC (in vitro assay) performed on Danish study subjects. Peripheral blood mononuclear cells PBMCs were isolated from 40 ml of heparinized blood by Lymphoprep (Nycomed Pharma A/S, Oslo, Norway) density centrifugation. Cells were washed twice in RPMI 1640 medium with 5% fetal calf serum and resuspended in RPMI 1640 medium supplemented with 10 mM HEPES, 20 U of penicillin per ml, and 20 μg of streptomycin (Gibco Ltd., Paisley, United Kingdom) per ml; 58.4 μg of L-glutamine per ml; and 15% heat-inactivated pooled normal human serum. Cell concentration was determined by microscopy in a Fuchs-Rosenthal counting chamber after crystal violet staining.

M. avium PPD and *M. bovis* PPD were used at a final concentration of 12 μg/ml. Tetanus toxoid (TT; Statens Seruminstitut, Copenhagen, Denmark) was used as IL-5 stimulation control at a final concentration of 3 μg/ml. PHA for nonspecific stimulation for proliferation assay was purchased from Difco and used at a final concentration of 40 μg/ml. Phorbol myristate acetate (PMA) was purchased from Sigma and used at a final concentration of 50 ng/ml. Ionomycin was purchased from Calbiochem (San Diego, Calif.) and used at a final concentration of 1.5 μg/ml.

PBMCs were adjusted to 2×10^6 cells/ml. One hundred microliters of cell suspension was incubated with 100 μl of antigen, mitogen, or medium for 7 days in round-bottomed microculture plates (Nunc, Roskilde, Denmark), at 37°C and 5% CO_2 in a humidified atmosphere. Cells in six wells were tested with each stimulating agent. Half of the stimulated wells were pulsed with PMA and ionomycin for the last 18 h before harvest of the supernatants for IL-5 determinations as previously described (47, 48). The supernatants were stored at −80°C for later cytokine determinations. Cytokines (IFN-γ and IL-5) were determined by ELISA (Genzyme, Cambridge, Mass.) following the manufacturer's protocol. Results are presented as antigen-induced cytokine production (micrograms per milliliter of culture supernatant), which is the total cytokine concentration measured in the antigen-stimulated culture minus that of the medium-only control.

Division of testing responsibilities. All resected bowel tissue homogenates from both U.S. and Danish patients and control subjects were tested by PCR for IS*900* in Denmark (U.S. and G.L.). All sera from both U.S. and Danish patients and controls were tested for antibodies to *M. avium* subsp. *paratuberculosis* in the United States (M.T.C.). Cultures on tissue samples were done by the same methodology, with minor variation, in both Denmark (U.S. and G.L.) and the United States (M.T.C.). Cytokine responses of PBMCs were determined using the ex vivo whole blood assay (M.T.C.) for U.S. subjects and by in vitro assay using purified PBMCs for Danish subjects (C.M.).

Blinded laboratories. All samples were coded by the individual in charge of specimen collection (S.C. in Denmark and D.C. in the United States), and the technologists performing the assays knew nothing about specimen sources beyond the six-digit specimen identification number. Only after samples from all controls and patients had been obtained and analyzed did the investigators decode the data matching patient disease status and clinical data with diagnostic test results.

Statistical analysis. Categorical data, e.g., PCR positive or negative, were evaluated by Fisher's exact test (two tailed), and both *P* values and odds ratios (OR) with 95% confidence intervals are reported. Test results providing continuous response variables were first tested to determine if the data from all comparison groups had equal standard deviations (Bartlett's test) and were from Gaussian distributions (method of Kolmogorov and Smirnov). If so, ordinary one-way analysis of variance (ANOVA) was done followed by Tukey's multiple comparison posttest. When the groups had unequal variances or were not Gaussian in distribution and data transformations failed to render the data Gaussian, the Kruskal-Wallis nonparametric ANOVA was used followed by Dunn's multiple comparisons test (GraphPad InStat version 3.00 for Windows 95; GraphPad Software, Inc., San Diego, Calif.). Rate differences on independent assays were analyzed using the z statistic (56), and when not independent, i.e., more than one assay done on the same patient, McNemar's test was used (55). Differences in test results between study groups are reported as significant when $P < 0.05$.

RESULTS

A total of 439 IBD patients and 324 control subjects participated in the study (Table 1). An additional 252 Red Cross blood donors served as controls in determining the ELISA cutoff. Almost the same number of IBD patients came from the United States and Denmark. Patients and controls in all study groups and subgroups were the same age except for the surgical controls in Denmark who were older ($P < 0.05$). Consistent with the majority of epidemiological studies (23), there were more females among CD patients (58.5%) than UC patients (48.5%) ($P < 0.05$). Over 78% of Danish subjects had been vaccinated with BCG, while only 4 (0.9%) American study subjects, all in the outpatient control group, reported a known history of BCG vaccination.

TABLE 2. PCR results for patients and controls from the United States and Denmark[a]

Type of result	CD		UC		Control	
	US	DK	US	DK	US	DK
Total no. of patients tested	30	49	35	26	18	30
Total no. of tissues tested	175	312	212	158	98	91
Mean ± SD (range) of tissues tested per patient	5.8 ± 2.1 (1–12)	6.4 ± 0.8 (4–7)	6.1 ± 1.4 (3–9)	6.1 ± 0.8 (3–7)	5.4 ± 1.6 (2–9)	3.0 ± 1.5 (2–7)
No. (range) of positive tissues per patient	0–4	0–3	0–3	0–2	0–1	0–1
No. (%) of total tissues positive	15 (8.6)	10 (3.2)	21 (9.9)	7 (4.4)	1 (1.0)	2 (2.2)
No. (%) of patients positive	9 (30.0)	6 (12.2)	12 (34.3)	4 (15.4)	1 (5.6)	2 (6.7)

[a] Abbreviations: US, United States; DK, Denmark. Significance: among U.S. groups, CD versus controls, $P = 0.07$; OR = 7.29 (0.84–63.38); UC versus controls, $P = 0.04$; OR = 8.87 (1.05–74.99); IBD versus controls, $P = 0.03$; OR = 8.11 (1.01–65.16); among Denmark groups, no significant differences; among U.S. and Denmark combined groups, CD versus controls, $P = 0.06$; OR = 3.52 (0.96–12.86); UC versus controls, $P = 0.01$; OR = 5.33 (1.45–19.59); IBD versus controls, $P = 0.02$; OR = 4.27 (1.24–14.67). Fisher's exact test, two sided, was used (95% confidence interval). The number tested was less than total number in study for some groups due to failure to acquire samples. The positive rates by group, shown as number positive/total number (percent), are 15/79 (19.0), 16/61 (26.2), and 3/48 (6.3) for CD, UC, and control groups, respectively. The positive rate overall for all IBD patients (CD plus UC) was 31 positive of 140 total (22.1%).

Culture. A total of 2,263 cultures were set up on resected tissues from 83 U.S. patients (mean = 27.3 cultures/patient; tissues were not cultured on four patients), and 1,722 cultures were set up on 574 resected tissue samples from 108 Danish patients (mean = 15.9 cultures/patient; two patients were cultured twice). Overall, contamination rates were similar in both the U.S. and Danish laboratories (13%), with the majority of contaminants growing in cultures without antibiotics. No *M. avium* subsp. *paratuberculosis* isolates were obtained from any patient in either country. In Denmark, *Mycobacterium gordonae* was isolated from six patients (one tissue in each case), and in the United States, *M. avium* complex mycobacteria were cultured from five IBD patients.

IS*900*. With the exception of the Danish surgical control patients, over five tissues on average were tested per patient (Table 2). Among PCR-positive patients, occasionally multiple tissues tested positive but most often positive PCR results occurred with only a single tissue per patient. Throughout the study, every negative PCR control consistently was negative. The proximal end of the affected resected bowel (tissue 2) gave the highest percentage of positive PCR results (20%). Collectively, >34% of positive PCR results were from lymph nodes. A remarkable difference between Danish and U.S. patients with respect to rate of positive PCR findings was found: 30, 34.3, and 5.6% of U.S. CD, UC, and control patients were positive, respectively, compared to 12.2, 15.4, and 6.7% of Danish CD, UC, and control patients, respectively ($P = 0.025$). As shown in Table 2, in the U.S. patient material, the difference in PCR-positive rate of IBD patients (CD and UC combined) and controls was significant ($P = 0.04$). Among Danish study groups, no significant differences in rate of PCR positivity were found. For the combined U.S. and Danish groups, rates of PCR-positive persons were 19.0, 26.2, 22.1, and 6.3% for CD patients, UC patients, all IBD patients, and control patients, respectively. PCR-positive rates for UC and IBD were significantly higher than controls ($P < 0.03$). The PCR-positive rate for U.S. CD patients and combined U.S. and Danish CD patient groups compared to their respective controls was not significant by two-tailed Fisher exact test ($P = 0.07$ and $P = 0.06$, respectively); however, by the one-tailed Fisher exact test and by the z statistic (for independent proportions), the PCR-positive rate for these CD patient groups was significantly higher than their respective controls ($P < 0.05$).

ELISA. Bovine negative control serum provided with the ELISA kit yielded slightly lower OD values than the negative control human serum (bovine = 0.088; human = 0.103). Bovine and human positive control sera did not produce significantly different ELISA OD values (bovine = 0.375; human = 0.357). For 251 Red Cross donor sera (one outlier excluded), the ELISA s/p mean and standard deviation were 0.065 ± 0.123 (mode = 0.050). Over 92% of these sera had s/p values of <0.25, the cutoff recommended by the kit manufacturer when bovine sera are tested. A positive test on human sera was defined based on Red Cross donor sera as the mean plus 2 standard deviations, i.e., s/p = 0.312, which by definition gave the assay a 95% specificity.

U.S. CD surgical patients had the highest rate of ELISA-positive individuals (20.7%) while UC surgical patients in both the U.S. and Danish populations had no ELISA-positive individuals (Table 3). The two strongest serological reactors were U.S. CD outpatients; both had ELISA s/p values of >1.8. Among U.S. study groups, the rate of ELISA-positive CD patients was higher than that of UC patients ($P = 0.005$) as well as controls ($P = 0.002$). Among Danish groups, there were no significant differences in ELISA-positive rates. When both surgical and outpatient subgroups from both the United States and Denmark were combined, the rate of ELISA-positive CD patients was not higher than for UC patients ($P = 0.089$), but it was much higher than for controls ($P = 0.0006$). When all IBD patients were considered as a group, the rate of ELISA-positive patients was higher than controls ($P = 0.003$). The rate of ELISA-positives in the study control population was not significantly different from that of the Red Cross donors used to establish the assay cutoff, serving to validate the choice of cutoff value for a positive test.

IFN-γ for whole blood assay. IFN-γ results in the ex vivo whole blood assay were much lower than those found after in vitro culture and stimulation of cultured PMBCs for Danish study subjects. Levels of IFN-γ release in response to both mycobacterial PPD preparations and PHA were significantly lower among both CD and UC surgical patients than controls ($P < 0.05$) (data not shown). Concerned about confounding influences of surgery and/or therapy that patients in these groups were undergoing prior to surgery, the subsequent analysis was restricted to the outpatients (Table 4). CD outpatients had lower IFN-γ levels in the ex vivo assay after exposure of leukocytes to *M. avium* PPD ($P < 0.01$), *M. bovis* PPD ($P < 0.001$), and PHA ($P < 0.01$). UC outpatients, in contrast, were not significantly different from controls.

IFN-γ release by purified PBMCs. IFN-γ release in response to PHA for the in vitro assay was lower for all surgical

TABLE 3. ELISA results for patients and controls from the United States and Denmark[a]

Type of result	CD				UC				Control			
	Surgical		Outpatient		Surgical		Outpatient		Surgical		Healthy	
	US	DK	US	DK	US	DK	US	DK	US	DK	US	DK
No. tested	29	47	113	72	34	25	31	74	17	28	195	79
No. positive (s/p >0.312)	6	3	13	8	0	0	1	9	1	2	5	4
% positive	20.7	6.4	11.5	11.1	0.0	0.0	3.2	12.2	5.9	7.1	2.6	5.1

[a] Abbreviations: US, United States; DK, Denmark. For all comparisons of surgical to matched outpatient group, no significant differences were found. Significance: among U.S. groups, CD versus controls, $P = 0.0002$; OR = 2.03 (1.57–2.64); UC versus controls, $P = 1.00$; OR = 0.60 (0.09–3.75); CD versus UC, $P = 0.005$; OR = 1.44 (1.25–1.69); among Denmark groups, no significant differences; among U.S. and Denmark combined groups, CD versus controls, $P = 0.0006$; OR = 3.32 (1.66–6.63); UC versus controls, $P = 0.26$; OR = 1.66 (0.70–3.93); CD versus UC, $P = 0.089$; OR = 2.00 (0.95–4.21); IBD versus controls, $P = 0.003$; OR = 2.66 (1.37–5.16). Fisher's exact test, two sided, was used (95% confidence interval). The number tested was less than total number in study for some groups due to failure to acquire samples. The positive rates by group, shown as number positive/total number (percent), are 30/261 (11.5), 10/164 (6.1), and 12/319 (3.8) for CD, UC, and control groups, respectively. The positive rate overall for all IBD patients (CD plus UC) was 40 positive of 425 total (9.4%). Combined positive percents (United States plus Denmark) were 11.8, 11.4, 0, 9.5, 6.7, and 3.3 for CD, UC, and control surgical and outpatient groups, respectively.

subgroups compared to outpatients ($P < 0.05$). Thus, just as for the U.S. patients, analysis of disease effects on IFN-γ release was restricted to the outpatient groups (Table 5). BCG vaccination status was found to significantly affect IFN-γ responses to both *M. avium* and *M. bovis* PPD ($P < 0.001$). For this reason and because few Danish study subjects were not vaccinated, non-BCG-vaccinated persons were excluded from group comparison analysis. IFN-γ responses to both PPD preparations as well as PHA were significantly lower for both CD and UC BCG-vaccinated outpatients ($P < 0.01$). In response to *M. avium* PPD, CD patient PBMCs released significantly less IFN-γ than did UC patient PBMCs ($P < 0.05$).

IL-5 release by purified PBMCs. Analysis was restricted to outpatients who were BCG vaccinated to limit effects of confounding factors (Table 6). With one exception, no differences were found among groups in IL-5 responses to either mycobacterial PPD used for TT. In response to TT, UC patient PBMCs showed a slightly lower level of IL-5 release than controls ($P < 0.05$).

Correlation between PCR and ELISA. For 181 study subjects, including CD and UC patients and controls, both PCR analysis of surgically resected bowel and lymph node tissues and ELISA for serum antibodies to *M. avium* subsp. *paratuberculosis* were performed. For 12 patients, either PCR or ELISA data were missing. Of the 181 subjects, 11 were ELISA positive, 34 were PCR positive, and 136 were negative for evidence of *M. avium* subsp. *paratuberculosis* infection by both tests. Interestingly, in no instance was an individual positive by both tests. McNemar's test for association when tests are not independent showed this to be highly significant ($P < 0.005$).

Correlation between PCR or ELISA and IFN-γ and IL-5. No significant association was found between ELISA-positive status or PCR-positive status and level of IFN-γ or IL-5 release after stimulation of PBMCs with either mycobacterial PPD.

Effect of BCG vaccination on ELISA, IFN-γ, IL-5, and PCR. BCG vaccination did not affect levels of serum antibody to *M. avium* subsp. *paratuberculosis*; however, BCG-vaccinated IBD patients had significantly higher IFN-γ levels after stimulation of PBMCs with either *M. avium* PPD or *M. bovis* PPD ($P < 0.001$). No effect of vaccination on IL-5 levels was seen (data not shown). A total of 15 Danish IBD patients tested by PCR were not BCG vaccinated. Of these, five (33.3%) tested PCR positive. This was significantly more than the 5 of 57 (8.8%) BCG-vaccinated Danish IBD patients who tested PCR positive ($P = 0.02$). Four Danish IBD patients had unknown BCG vaccination status and were excluded from the analysis. All were PCR negative.

TABLE 4. IFN-γ levels in plasma after stimulation of whole blood with mycobacterial antigens or PHA for U.S. outpatient study groups[a]

Antigen or mitogen	Value type	Level (pg/ml) for group (*n*):		
		CD (101)	UC (31)	Control (169)
M. avium PPD	Median	0[b]	1.0	1.5
	Mean ± SEM	7.1 ± 2.4	3.5 ± 1.3	9.7 ± 2.3
	Range	0–209	0–29	0–321
M. bovis PPD	Median	0[c]	1.0	1.0
	Mean ± SEM	16.5 ± 8.9	2.2 ± 0.8	6.3 ± 1.5
	Range	0–776	0–24	0–189
PHA	Median	831[b]	963	1,059
	Mean ± SEM	731 ± 49	744 ± 80	864 ± 33
	Range	0–1,600	0–1,200	0–1,600

[a] Kruskal-Wallis nonparametric ANOVA with Dunn's multiple comparisons test. The UC group was not different from controls for any antigen or mitogen.
[b] CD values were lower than controls, $P < 0.01$.
[c] CD values were lower than controls, $P < 0.001$.

DISCUSSION

The insertion element IS*900* is unique to *M. avium* subsp. *paratuberculosis* (15, 49, 74). Resected bowel and lymph node tissues from IBD patients were found to be IS*900* positive by PCR significantly more often than controls (Table 2). Over one-third of positive tissues were lymph nodes, indicating that the infection was deeper than the mucosa. Higher PCR-positive rates were observed in U.S. patients than in Danish patients ($P = 0.025$). This difference appeared to be related to the different rates of BCG vaccination among study subjects between the United States and Denmark, an observation substantiated by the much higher rate of PCR-positive Danish IBD patients among non-BCG vaccinates (33.3 versus 8.8%). The rate of PCR-positive U.S. CD patients (30%) is consistent with the majority of similar previous studies that used resected bowel tissue rather than biopsy samples (18, 27, 45, 49, 59). The finding that U.S. UC patients also were PCR positive more often (34.4%) than control subjects (5.6%) was unexpected but similar to some such previous studies (46, 52).

The IS*900* sequence detected was in low abundance. Band intensity of the amplified product on gels was much weaker on human tissues than for animal tissue controls. Few tissues, most often only one, were PCR positive among PCR-positive patients. Studies testing fewer tissues, smaller pieces of tissue, e.g., biopsy samples, or only histopathology tissue sections

TABLE 5. IFN-γ levels in PBMC culture supernatants after stimulation with mycobacterial antigens or PHA for Danish outpatient study groups, excluding those not BCG vaccinated[a]

Antigen or mitogen	Value type	Level (pg/ml) for group (*n*):		
		CD (56)	UC (64)	Control (63)
M. avium PPD	Median	1,860**	5,326**	9,303
	Mean ± SEM	3,837 ± 644	6,519 ± 766	9,829 ± 761
	Range	1–19,309	1–30,073	1–21,327
M. bovis PPD	Median	2,243**	4,176**	10,734
	Mean ± SEM	4,628 ± 717	6,053 ± 771	11,685 ± 1,085
	Range	1–18,245	1–29,542	1–46,100
PHA	Median	10,492**	9,468**	15,631
	Mean ± SEM	10,144 ± 740	9,635 ± 654	14,598 ± 801
	Range	482–19,425	1–19,662	1–25,932

[a] Kruskal-Wallis nonparametric ANOVA with Dunn's multiple comparisons test. For IFN-γ release after *M. avium* PPD stimulation, CD < UC ($P < 0.05$). For IFN-γ release after *M. bovis* PPD or PHA stimulation, CD and UC groups were not significantly different. Asterisks denote comparison of CD and UC groups to controls: *, $P < 0.01$; **, $P < 0.001$.

would likely have found lower rates of PCR-positive patients and controls, possibly explaining negative results for IS*900* PCR reported in other recent studies (2, 7, 22, 28, 57).

Although molecular evidence indicates the presence of *M. avium* subsp. *paratuberculosis* in tissues of CD and UC patients, all cultures for this organism from the same tissues tested by PCR, using methods shown to be sensitive in isolation of *M. avium* subsp. *paratuberculosis* from animals, were negative. The methods employed limited exposure of tissues to the HPC decontaminant, and for every tissue one culture was done in media without antibiotics, thus limiting potential interference with *M. avium* subsp. *paratuberculosis* growth, even if it were in the cell-wall-deficient state as suggested by some investigations (50, 75). Concurrent with the present study, the U.S. laboratory (M.T.C.) made isolation of *M. avium* subsp. *paratuberculosis* from clinical samples submitted from a wide variety of animal species, including at least one primate, thus validating the efficacy of the culture system used for animal-origin strains of *M. avium* subsp. *paratuberculosis*. Both the Danish and American laboratories made sporadic isolations of other mycobacteria, substantiating their ability to isolate such organisms. These data suggest that the organism detected by PCR in IBD patients either had died, leaving behind its molecular footprint (IS*900*), or was in a viable but nonculturable state. However, other investigators have reported successful isolation of *M. avium* subsp. *paratuberculosis* from CD patients (9, 19).

TABLE 6. IL-5 levels in PBMC culture supernatants after stimulation with mycobacterial antigens or PHA for Danish outpatient study groups, excluding those not BCG vaccinated[a]

Antigen or mitogen	Value type	Level (pg/ml) for group (*n*):		
		CD (56)	UC (64)	Control (63)
M. avium PPD	Median	12.5	1.0	1.0
	Mean ± SEM	141 ± 31	117 ± 33	72 ± 22
	Range	1–941	1–1,538	1–1,097
M. bovis PPD	Median	3.0	1.0	1.0
	Mean ± SEM	213 ± 49	73 ± 16	144 ± 82
	Range	1–1,522	1–509	1–5,181
TT	Median	245	195*	532
	Mean ± SEM	555 ± 85	535 ± 96	698 ± 74
	Range	1–2,126	0–4,508	1–1,995

[a] Kruskal-Wallis nonparametric ANOVA with Dunn's multiple comparisons test. *, among all group comparisons for all antigens, only IL-5 release by UC patient PBMCs in response to TT was lower than controls ($P < 0.05$).

At the General Meeting of the American Society for Microbiology in 2000, Schwartz et al. reported that BACTEC 12B medium did not support growth of *M. avium* subsp. *paratuberculosis* from humans while BACTEC MGIT medium did, suggesting that human strains may be more fastidious in growth requirements than animal strains (D. Schwartz, I. Shafran, C. Romero, and F. A. Naser, Abstr. 100th Gen. Meet. Am. Soc. Microbiol. 2000, abstr. U-54, 2000).

The *M. avium* subsp. *paratuberculosis* ELISA found results similar in many respects to those of the IS*900* PCR: U.S. IBD patients were ELISA positive more often than their Danish counterparts (Table 3). This suggests that a confounding factor between these two populations affects both PCR and ELISA findings. Among study groups in Denmark, no significant differences in ELISA-positive rates were found. Among U.S. IBD patients, CD patients were ELISA positive significantly more often than controls ($P = 0.0002$; OR = 5.3; 1.57 to 2.64 [confidence interval]) but also more often than UC patients ($P = 0.005$; OR = 1.44; 1.25 to 1.69), supporting the findings of other studies (24–26, 64, 73). Support for serological evidence of mycobacterial infection in CD patients also comes from the recent work of Cohavy et al. (14). They described a novel 214-amino-acid iron-regulated mycobacterial protein called HupB, found that monoclonal antibodies to pANCA reacted with this protein, and reported that CD patients had higher serum levels of anti-HupB immunoglobulin A IgA than UC patients or controls.

Intriguing was the observation that among 181 Danish and American study subjects tested both by PCR for IS*900* and by ELISA for serum antibody to *M. avium* subsp. *paratuberculosis* in no case was a person positive by both tests ($P < 0.005$). This suggests that the presence of *M. avium* subsp. *paratuberculosis* and production of serum antibodies to the organism are mutually exclusive. This is the reverse of the situation found in multibacillary and paucibacillary leprosy (35, 69) as well as paratuberculosis in cattle and sheep where elevated numbers of *M. avium* subsp. *paratuberculosis* organisms in tissues and feces are associated with higher levels of serum antibody (12, 53, 61, 65).

The ex vivo assay for IFN-γ release after PPD exposure used on U.S. study subjects proved not to be very sensitive. Although peripheral blood leukocytes from most subjects released relatively high levels of IFN-γ in response to PHA (>700 pg/ml of plasma), IFN-γ levels after PPD stimulation were generally very low or undetectable (Table 4). However, in comparison to controls IFN-γ responses to PHA were signifi-

cantly lower in CD patients ($P < 0.01$) and slightly lower in UC patients, suggesting a possible influence of the disease or the immunosuppressive drug treatments given most of these patients. Hence, the results should be interpreted with caution. With these caveats in mind, we found that CD patients but not UC patients had significantly lower IFN-γ responses to PPD than controls by ex vivo leukocyte stimulation assays ($P < 0.01$). This observation was corroborated by in vitro assays done using cultured PBMCs from Danish patients. In cattle with late-stage clinical paratuberculosis, Koets et al. demonstrated a switch from Th1- to Th2-type T-cell reactivity that appeared to be associated with a loss of Th1-like T cells both at the site of infection and in circulation (38). Their data suggest *M. avium* subsp. *paratuberculosis*-infected macrophages induce apoptotic death of T cells.

Among Danish outpatients and controls, homogeneous for BCG vaccination status, IBD patients as a group had lower IFN-γ responses to PHA than controls, as was seen for U.S. IBD patients (Table 5). This again suggests that IBD patients are hyporesponsive due either to their disease or to the immunosuppressive drugs that these patients were being treated with (6). Among IBD outpatients, CD patients had lower IFN-γ levels in response to *M. avium* PPD than UC patients ($P < 0.05$). Steroid use differed significantly between these two groups also: 44.4% of CD outpatients and 20.3% of UC outpatients were on steroids in the month preceding, as well as at the time of, blood sample collection ($P = 0.02$).

Although IFN-γ responses were highly variable among patients and controls and potentially influenced by immunosuppressive drug therapy, when results of testing all U.S. and Danish IBD patients are considered collectively, exposure of leukocytes to an *M. avium* PPD preparation resulted in lower levels of IFN-γ release in CD patients than UC patients. When these results are considered in light of the positive PCR findings for patients with both diseases and ELISA-positive results seen primarily in CD patients, it suggests that CD and UC patients may differ primarily by the nature of their immune response to *M. avium* subsp. *paratuberculosis* or some similar mycobacterial agent.

No consistent effect of disease status on IL-5 release by PBMCs was observed between IBD patients and controls, nor between CD and UC patients (Table 6). However, there was a trend toward higher IL-5 levels in response to *M. bovis* or *M. avium* PPD stimulation in CD patients. The generally low and highly variable IL-5 levels observed precluded meaningful assessment of the Th1-Th2 immune response status of study subjects by IFN-γ/IL-5 ratios, and the negative influences of immunosuppressive drug therapy and positive effects of BCG vaccination further complicated this type of data analysis.

One other intriguing observation about cytokine patterns was made. Comparison of both IFN-γ and IL-5 responses to *M. bovis* and *M. avium* PPD preparations for the relatively few non-BCG-vaccinated CD and UC patients using scattergrams showed that UC patient PBMCs preferentially responded to *M. bovis* over *M. avium* while CD patient PBMCs responded equally well to both mycobacterial antigen preparations. This suggests that there is a difference between CD and UC patients in immune reactivity to mycobacterial antigens.

Support for mycobacterial involvement in IBD comes from the unexpected finding that BCG vaccination was associated with a lower rate of PCR-positive findings for IBD patients between the United States and Denmark (29.6 versus 13.3%) and within the Danish IBD patient population (33.3% for non-BCG vaccinates versus 8.8% among BCG vaccinates). To the best of our knowledge, no studies have evaluated whether BCG vaccination influences the incidence of IBD. Although comparison may not be valid, the incidence of CD and UC in the United States and Denmark is very similar (42, 51). BCG vaccination clearly induced PBMCs that responded to PPD exposure by release of very high levels of the proinflammatory cytokine IFN-γ, offering a plausible explanation for the apparent protective effect of BCG vaccination for *M. avium* subsp. *paratuberculosis* infection (IS*900* positivity). However, BCG is a potent nonspecific immune stimulator, and the observed association of BCG vaccination status and IS*900* PCR-positive status could be related to non-mycobacterium-specific factors.

Other differences between the U.S. and Danish populations could also potentially have affected *M. avium* subsp. *paratuberculosis* diagnostic results, in particular the predominantly urban origin of Danish study subjects in contrast to the more rural origin of U.S. (Wisconsin) study subjects. Although subjects from both countries were not systematically surveyed, 32% of the U.S. study participants responded "yes" to the question "Have you ever lived or worked on a dairy farm?" Dairy cattle have the highest known prevalence of *M. avium* subsp. *paratuberculosis* infections among animals. A more detailed epidemiologic analysis for association between *M. avium* subsp. *paratuberculosis* diagnostic results in this study and exposure risk factors as measured by questionnaire is the subject of a separate study.

In spite of several study limitations, a pattern of *M. avium* subsp. *paratuberculosis* diagnostic test results emerged that supports possible mycobacterial involvement in IBD, in particular CD. However, the pattern of diagnostic findings is admittedly complex and best appreciated in the context of certain other chronic infectious diseases that clinically present in more than one form due either to temporal changes in diagnostic results during infection progression, as in bovine paratuberculosis (37, 38, 63), and syphilis in humans (70), or to polarized immune responses as seen in lepromatous and tuberculoid leprosy (20, 21, 62), miliary and primary tuberculosis (3), or murine leishmaniasis (34, 43).

Investigation of the role that mycobacteria play in the pathogenesis of IBD may provide fruitful avenues for future studies. However, the complex interplay of potential age-dependent effects on susceptibility to *M. avium* subsp. *paratuberculosis* (as seen in cattle) and host genetic effects on infection susceptibility and immune response, as seen in many mouse models of chronic infectious diseases, as well as exposure to *M. avium* subsp. *paratuberculosis* or other risk factors, must be taken into consideration in study designs (1, 5, 29, 30, 40, 60). Further complicating such studies is the need to control for effects of immunosuppressive drug therapy and BCG vaccination on immunological assays. In addition to stimulating new directions for pathogenesis research in IBD, the theory of mycobacterial involvement provides interesting opportunities for treatment of IBD modeled after successful multidrug therapy for leprosy using efficacious antimicrobial agents in concert with cytokines or cytokine antagonists to modulate the host immune response as the infective agent is destroyed.

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