

Occurrence, in Crohn's Disease, of Antibodies Directed against a Species-Specific Recombinant Polypeptide of *Mycobacterium paratuberculosis*

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Sera from patients with Crohn's disease and controls were analyzed by an enzyme-linked immunosorbent assay based on the *Mycobacterium paratuberculosis*-specific recombinant polypeptide a362. Anti-a362 immunoglobulin G (IgG) ($P < 0.05$) and IgA ($P < 0.001$) titers were higher in patients with Crohn's disease than in controls. A monomodal Gaussian distribution of anti-a362 IgA levels was found for controls, and a bimodal distribution was found for patients with Crohn's disease. An *M. paratuberculosis* etiology is suggested for the 36% of patients with Crohn's disease who had an anti-a362 IgA level higher than that of controls.

Clinical symptoms of Crohn's disease, a chronic human enteritis of undefined etiology, mimic those of Johne's disease of ruminants, which is induced by *Mycobacterium paratuberculosis*. Granulomas and lymph node alterations in biopsy specimens from patients with Crohn's disease resemble those of bovine paratuberculosis (2, 3), hence the hypothesis of a common mycobacterial etiology. Although no acid-fast bacteria were found in granulomas from patients with Crohn's disease, prolonged incubation of some biopsy materials yielded *M. paratuberculosis* colonies (4). The etiology problem was also approached by exploring the humoral immune response of patients with Crohn's disease to mycobacterial antigens, but this approach has led to conflicting results. Serological assays based on glycopeptidolipids and lipoarabinomannan of *M. paratuberculosis*, on the A60 antigen complex of *M. bovis*, and on mycobacterial sonicates failed to show differences between the immunoglobulin (Ig) titers of patients with Crohn's disease and controls (5, 10, 11, 18). On the other hand, increased serological reactivities to mycobacterial antigens were reported by others, especially when IgA was analyzed (8, 16, 17, 20). However, in the cited works, serological assays were not based on purified *M. paratuberculosis*-specific antigens. We have recently shown that a 34-kDa protein of the *M. paratuberculosis* A36 complex is immunodominant in bovine paratuberculosis and contains B-cell epitopes specific for all tested mycobacteria (6, 7). The gene coding for the 34-kDa protein was isolated by molecular cloning and sequenced. The 897-bp open reading frame coded for a novel protein containing well-defined hydrophobic and hydrophilic portions (9). A DNA fragment coding for the hydrophilic carboxyl end of the 34-kDa protein has been isolated from a λ gt11 genomic library of *M. paratuberculosis*. This DNA fragment coded for a polypeptide (a362) containing B-cell epitopes present in all tested *M. paratuberculosis* strains (including a strain isolated from a patient with Crohn's disease) but not in related bacteria, including strains of the *Mycobacterium avium*-*M. intracellulare*-*M. scrofulaceum* group. The specificity and antigenicity of the a362 polypeptide prompted its use in an enzyme-linked immunosorbent assay

(ELISA) for paratuberculosis. This assay proved to correctly identify paratuberculous animals on the basis of tested blood samples from healthy and infected cattle (7). In the present study, such an a362-based ELISA was used to compare the serological responses of patients with Crohn's disease with those of healthy controls.

The a362 recombinant polypeptide was prepared as a fusion with the 25 first amino acids of mouse tumor necrosis factor, as previously described (9). The corresponding vector was used to avoid the inconveniences of the usual recombinant products fused with the β -galactosidase gene product of *Escherichia coli*. This polypeptide was used (0.5 μ g of a362 in 100 μ l of 0.05 M sodium carbonate buffer [pH 9.6] per well) to coat microtiter plates (Microwell Module, high binding capacity; Nunc, Roskilde, Denmark). The plates were air dried overnight, saturated (300 μ l of 0.1% [wt/vol] serum albumin-0.15 M NaCl solution per well, 1 h, 37°C), and used for serological analysis of 50 healthy subjects and 73 patients with Crohn's disease with matching age and sex distributions. Healthy subjects were blood donors from the Belgian Red Cross Organization, and patients with Crohn's disease were diagnosed at the St Luc University Clinics (Brussels, Belgium) by conventional clinical, radiological, endoscopic, and histological criteria. After incubation of sera (100 μ l per well, 1 h, 37°C) diluted to the optimum dilution of 1/50 in PBST buffer (0.15 M NaCl, 0.02 M phosphate buffer [pH 7.2], 0.005% Tween 80), washing with PBST, and addition of peroxidase-conjugated rabbit anti-human IgG or IgA (Dako, Copenhagen, Denmark) (100 μ l of a 1/5,000 dilution in PBST, 1 h, 37°C), the excess reagent was removed by PBST washings, and peroxidase substrate was added (100 μ l of 17 mM sodium citrate buffer [pH 6.3] containing 0.2% [wt/vol] *o*-phenylenediamine and 0.015% [wt/vol] hydrogen peroxide, 30 min, 37°C, in the dark). The reaction was stopped by addition of 100 μ l of 2 M H₂SO₄ per well, and the A₄₉₂ was measured with an SLT 210 reader (Kontron Analytical, Watford, United Kingdom). A statistical comparison of ELISA data from the two populations was done by the nonparametric Mann-Whitney U test (Instat 1.14; GraphPAD Software, Inc., San Diego, Calif.). Patients with Crohn's disease had significantly higher anti-a362 IgG ($P < 0.05$) and IgA ($P < 0.001$) titers than controls (not shown). Anti-a362 IgA titers were then analyzed by mixture population

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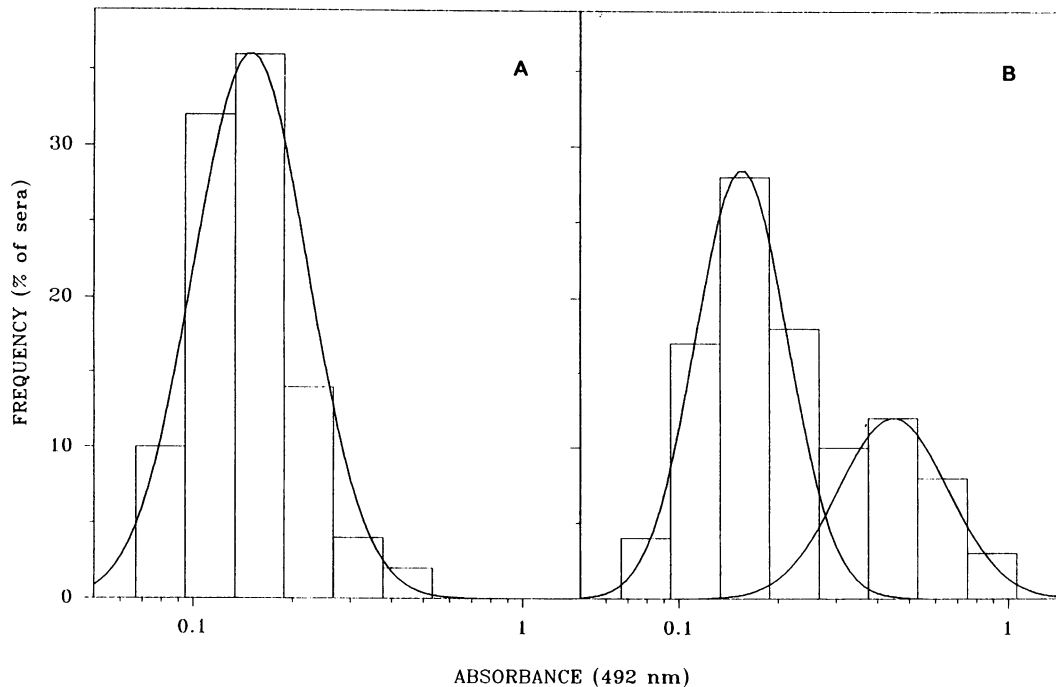


FIG. 1. Comparative distribution of antimycobacterial IgA titers in sera from patients with Crohn's disease and controls. Sera from 50 healthy individuals (A) and 73 patients with Crohn's disease (B) were analyzed by the a362 ELISA. Sera were distributed in frequency classes of 0.15 logarithmic unit of optical density (A_{492}) (bars), and the Gaussian curves best fitting the experimental data were computed (continuous curves).

modelling (14). The sera were clustered, according to their ELISA absorbances, in frequency classes of 0.15 logarithmic unit of optical density (A_{492}) (Fig. 1). The numbers of populations included in our data set, with optimal means and standard deviations, were estimated by the optimal likelihood function. A Gaussian distribution was then reconstituted for each population (Fig. 1).

A monomodal Gaussian distribution of control values, compatible with a single population, was observed (Fig. 1A), whereas the bimodal distribution of sera from patients with Crohn's disease supported the occurrence of two populations (Fig. 1B). In 64% of patients with Crohn's disease, the anti-a362 IgA level was comparable to that of controls (mean = 0.149 and 0.154, respectively) whereas titers were significantly higher in 36% of the patients (mean = 0.450).

The search for a higher level of antibody directed against mycobacteria in patients with Crohn's disease was not always successful (5, 10, 11, 18). Because of the presence of different mycobacteria in the intestinal tract, humoral immune responses to mycobacterial antigens are likely to occur. The use of the species-specific a362 assay would lower the otherwise high background level and restrict the response to a specific target. Moreover, IgA should be preferentially involved in such enteropathies as Crohn's disease, and differences with respect to controls are expected to be more evident whenever a study is restricted to a specific single compound. Several hypotheses can be proposed to explain the occurrence of two subpopulations among patients with Crohn's disease (Fig. 1B). Crohn's disease might include forms with similar symptoms but different etiologies. In the case of Crohn's disease patients with high anti-a362 IgA titers (one-third of the patients in this study), *M. paratuberculosis* may be the etiological agent. In agreement with an *M. paratuberculosis* etiology, hybridization of species-specific nucleic acid probes with the DNA of isolates amplified

by the PCR has allowed the identification of *M. paratuberculosis* in biopsy specimens from 65% of patients with Crohn's disease, compared with the 12.5% positivity rate of healthy persons (15, 19). In other cases, mycobacteria such as *M. kansasii* or *M. tuberculosis* might be responsible for some of the 64% of Crohn's disease cases diagnosed as negative by the a362 test, which is species specific (2, 13). On the other hand, the level of anti-*M. paratuberculosis* Ig may be low in some stage of the disease, as is the case for tuberculoid leprosy and primary tuberculosis (1, 12). Finally, sera of some patients with Crohn's disease may recognize mycobacterial B-cell epitopes different from those present in the a362 polypeptide, on which our assay is based.

In conclusion, our work shows the presence of Ig directed against a species-specific antigen of *M. paratuberculosis* in 36% of the patients with Crohn's disease examined, thus implicating this mycobacterium in the etiology of Crohn's disease.

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