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Antibodies to *Mycobacterium paratuberculosis*-specific protein antigens in Crohn's disease

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SUMMARY

The possible role of infection with *Mycobacterium paratuberculosis* (MAP) for the etiopathogenesis of Crohn's disease (CD) has been a matter of long-term controversy. In addition to similarities with the pathology of ruminant paratuberculosis, DNA fingerprinting confirmed the organism isolated from gut tissue, but the specificity of the immune repertoire has not as yet been evaluated. We report here on a serological study of 29 patients with CD, 20 patients with ulcerative colitis and 18 healthy control subjects, using three antigens attributed with species-specificity and selective immunogenicity following MAP infection. Antibodies binding to the 38-kD band of MAP extract were demonstrable by the Western blot technique in 57% of CD patients. Antibody levels to the 24-kD (p24BCD) cathodic bands, determined by competition ELISA using a monospecific murine antiserum, and to the 18-kD protease-resistant purified bacterioferritin, detected by standard ELISA, were significantly elevated in 53% of CD patients. However, these three antibody specificities tested in individual CD patients did not show any correlation with each other. Thus, 18% of patients were positive for all three specificities, whilst 84% had antibodies to at least one of the specific antigens. Although the exact proportion of affected patients is yet to be defined, the serological results obtained support the view that MAP infection may play an etiological role in Crohn's disease.

Keywords mycobacteria paratuberculosis antibodies Crohn's disease serology

INTRODUCTION

Inflammatory bowel diseases, particularly Crohn's disease (CD), has been suspected for many years of having a mycobacterial association [1,2]. This has to a large extent been based on similarities of the pathological and clinical manifestations of Johne's disease in ruminants which is known to be caused by *Mycobacterium avium ss paratuberculosis* (MAP). Supportive evidence of positive MAP bacterial culture from gut specimens [3] has been confirmed by DNA fingerprinting using the MAP-specific IS900 insertion sequence [4]. Using this DNA probe, positive samples of intestinal biopsy were found in 40% of CD patients, but only in 4·3% of ulcerative colitis (UC) and 12·5% of control subjects [5]. These results have been interpreted as consistent with an etiological role for MAP in a significant proportion of patients with CD.

Further evidence for an association of MAP infection with CD on the grounds of immune reactions to MAP-specific antigenic determinants would require the exclusion of cross-reactivity with the closely related commensal organisms of the *M. avium* group [6]. Previous serological studies reported elevated antibody levels in a certain proportion of CD patients against crude whole MAP extract [7,8], or the 65-kD heat-shock protein of *M. tuberculosis* [9,10]. MAP antigens were identified using antisera from animals with Johne's disease and from immunized rabbits using crossed immunoelectrophoresis [11], and recombinant DNA expression libraries [12,13]. However, gene cloning with MoAbs has as yet only been carried out with *M. avium*-specific antigens [14,15]. Recently, bacterioferritin and a 31-kD antigen A, homologous to the BCG85C complex, have been identified in MAP and found to be of high serological specificity and sensitivity for ovine paratuberculosis [16,17].

The background to this study is the analysis of murine antibody responses following infection with MAP and with two distinct strains of *M. avium* [18]. On the basis of selective immunogenicity, three antigens, characterized by the molecular weights of 38, 24 and 18 kD, have been selected for serological analysis with the human sera. Of these antigens, the 38-kD seems to be related to a major immunogen of tubercle bacilli [19–21], the 24-kD antigens represented by at least two distinct constituents have no proven relationship to any of the mycobacterial proteins of known structure, whereas the 18-kD protease-resistant antigen probably corresponds to the MAP bacterioferritin [16].

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MATERIALS AND METHODS

Sera

Serum samples were collected at the 'Nuovo Regina Margherita' Hospital, Italy, from 29 patients with CD and 20 patients with UC who had their diagnosis confirmed by clinical, radiological and histological criteria. Patients did not have a past history of either tuberculosis, or contact with active cases in the family. The disease activity and pathology varied widely between patients. Tuberculous enteritis was excluded and the differential diagnosis of CD, or UC, was based on histological, endoscopic and/or radiological evidence. All patients were receiving 10–50 mg corticosteroids daily. The control group was represented by 18 normal healthy individuals (NH), all bacille Calmette–Guerin (BCG)-vaccinated.

Serum from a goat with clinical signs of Johne's disease, confirmed by post-mortem pathology and bacteriological evidence of infection with *M. paratuberculosis*, was kindly donated by Professor I. McConnel and Dr A. Colston from the Department of Veterinary Pathology, Edinburgh, UK.

Mycobacterial extracts

M. avium ss paratuberculosis, strain 203, a recent isolate from cattle with Johne's disease, was grown on modified Dubos medium in the presence of 2 μg/ml mycobactin J (Allied Laboratories Inc., Lyons, France). The MAP-soluble extract (MPSE) was prepared from gamma-irradiated bacteria, by homogenization with glass beads, as described previously for tubercle bacilli [21]. After centrifugation for 1 h at 100 000 ***g***, the supernatants containing 1–2 mg protein per ml were stored in small aliquots at −20°C. Soluble extracts from *M. tuberculosis* (MTSE) and from other mycobacteria were prepared by the same procedure.

Gel electrophoresis

Soluble antigenic extracts (e.g. MPSE) were separated under reduced conditions by SDS–PAGE in 15% w/v final acrylamide concentration at 17 mA per gel for 60 min. Two-dimensional electrophoresis was performed with the MPSE treated with sample buffer containing 9 M urea and 10% Nonidet P40 [22]. Following isoelectric focusing in tube gels using 4% ampholytes in pH range 5–7 and 1% ampholytes of pH 3·5–10 (LKB Pharmacia, Uppsala, Sweden), separation in the second dimension was carried out by SDS–PAGE as described above.

For Western blotting, the proteins separated by either one- or two-dimensional gel electrophoresis were transferred to nitrocellulose membranes by electrophoresis at 10 V for 20 h. The nitrocellulose strips were incubated at room temperature with 1:100 diluted goat or human sera and developed with either anti-goat or anti-human IgG respectively.

Purified antigens

The 18-kD antigen was purified from MPSE after digestion with proteinase K (BDH Chemicals). The extract mixed with the enzyme solution in saline at a final concentration of 0·7 mg/ml was incubated for 30 min at 60°C. Subsequently, the digest was passed through a Sephadex G-50 column (3 × 65 cm; Pharmacia, Uppsala, Sweden) equilibrated with PBS and the fractions containing the protease-resistant material were collected and concentrated. The resulting material produced a single 18-kD band stained by Coomassi blue following separation by SDS–PAGE. A corresponding band developed by the serum from MAP-infected BALB/c mice was detected by Western blot analysis.

The anodic antigen of 24-kD molecular weight (p24A) was purified by two-dimensional gel electrophoresis. Following two-dimensional separation, the gel at the predetermined position [18] was cut out and the protein was eluted by incubation in 1 ml of 5 M urea for 20 h at 4°C and dialysed against PBS, pH 7·4. A single 24-kD band was detected by silver staining following separation of the eluted fraction by SDS–PAGE.

ELISA

Microtitre plates (Nunc-Immuno Plate MaxiSorp F96) were coated with 50 μl of purified p24A or protease-resistant antigen at a concentration of 2 μg/ml and blocked with 2% dried milk (w/v) in PBS containing 0·05% Tween 20. Plates were incubated with four-fold dilutions (1:50–1:3200) of human sera (50 μl) for 60 min at 37°C, followed by goat anti-human IgG peroxidase conjugate (Sigma) and the colour was developed with 0·1 mg/ml tetramethyl benzidine tetrahydrochloride (Aldrich, Gillingham, UK). The absorbance at 450 nm was read in the Titertek Multiskan II spectrometer. Antibody titres were expressed as the dilution of serum giving 30% of the plateau binding value of a standard positive serum (ABT_{30}).

ELISA-competition assay

This test, based on the inhibition of antigen binding of the reference mouse serum of selected specificity, allowing the titration of antibodies of corresponding epitope specificity in the human test sera, was performed as described previously [23]. Briefly, plates were coated with 50 μl of MPSE at concentration of 10 μg/ml. After blocking, serially diluted human sera were added to the wells and incubated for 2 h at 37°C. Without removal of liquid, 50 μl of the pooled serum from MAP-infected C57/Bl mice [18] were added to the wells at a 1:300 dilution that gave 80% of the maximum binding to MPSE for a further 1 h. Washed plates were incubated with goat anti-mouse IgG peroxidase conjugate and the colour was developed as above. The results were expressed as antibody titre giving 50% binding inhibition of the mouse sera (ID_{50}).

RESULTS

Response to the 38-kD antigen

Western blot analysis of CD sera showed antibody binding to several bands against the separated MPSE. Although the binding patterns varied greatly between individual sera, representative Western blots from three patients demonstrate that anti-38-kD antibodies were consistently detected in separate bleeds taken from each patient at intervals 5 months apart (Fig. 1). Altogether, the 38-kD band was detected in 16 out of 28 (57%) CD sera tested.

The specificity of the anti-38-kD antibodies in CD sera was analysed by Western blots of soluble extracts of several species of mycobacteria (Fig. 2). Sera from CD patients, selected for analysis on the basis of strong reactivity with the 38-kD band of MPSE (lane 6) reacted to a lesser degree with the 38-kD band of *M. avium*, strain RFLP-A6 (lane 4), but did not show a 38-kD band in extracts from *M. avium* strain SJ-B2, *M. scrofulaceum*, *M. kansasii* and *M. tuberculosis* (lanes 1, 2, 3 and 5). Western

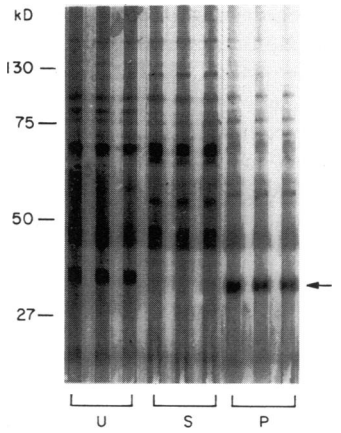

Fig. 1. Western blot analysis of sera from Crohn's disease (CD) patients. Bands separated from soluble extract of *Mycobacterium paratuberculosis* were developed with 1:100 diluted sera collected at three separate occasions within a 5-month interval from three patients (U, S, and P) with CD. The position of the 38-kD molecular weight band is marked by the arrow.

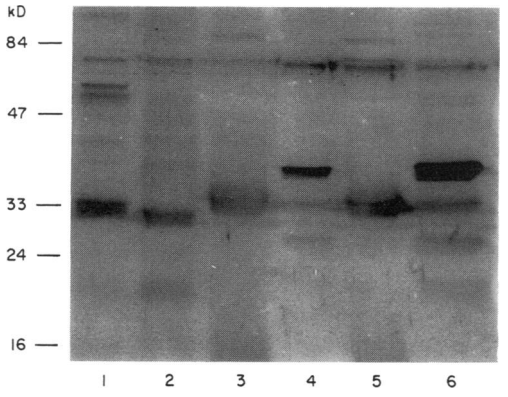

Fig. 2. Species specificity of the 38-kD antigen detected by Crohn's disease (CD) serum. Serum from patient U (see Fig. 1), diluted at 1:100, reacted with antigens separated from soluble extracts of: 1, *Mycobacterium tuberculosis*; 2, *M. kansasii*; 3, *M. scrofulaceum*; 4, *M. avium* strain RFLP-A6; 5, *M. avium* strain SJ-B2; and 6, *M. paratuberculosis* strain MAP-203.

blot analysis of all 28 CD sera confirmed the absence of a 38-kD band in MTSE (results not shown).

Analysis of response to diverse 24-kD constituents

Our interest in this constituent is based on the recent finding that antibodies from sera of MAP-infected C57Bl/6 mice reacted almost exclusively against a 24-kD band, shared between MAP and *M. avium* [18]. Furthermore, it was shown that antibodies from MAP-infected mice reacted with a cathodic band, whereas sera from *M. avium* (strain B2)-infected mice reacted with an anodic band of the B2 strain soluble extract.

Present analysis of the antigenic constituents of MPSE separated by two-dimensional gel electrophoresis (Fig. 3a) showed that the serum from a goat with paratuberculosis detected the 24-kD anodic band (A), as well as three bands with cathodic mobility (B, C, D). The goat serum detected the anodic

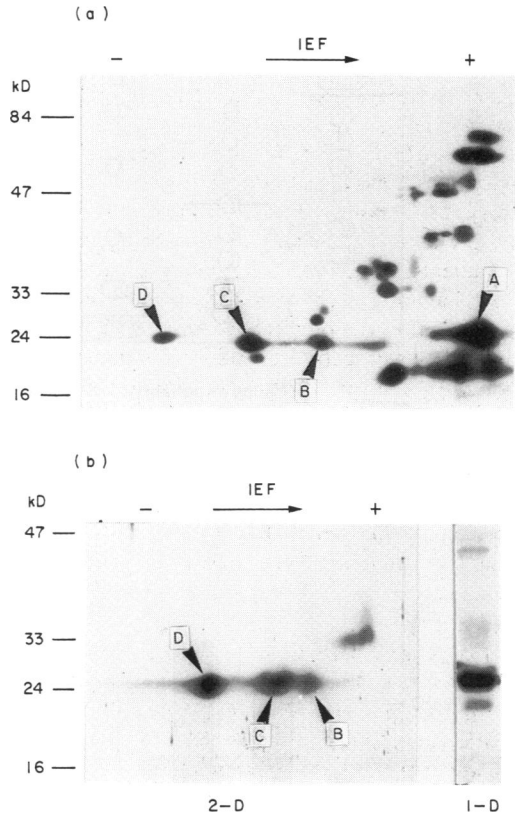

Fig. 3. Analysis of the 24-kD antigenic constituents of *Mycobacterium paratuberculosis*. (a) *Mycobacterium paratuberculosis*-soluble extract (MPSE) antigens separated by two-dimensional gel electrophoresis were developed by 1:100 diluted serum from a goat affected by paratuberculosis. The 24-kD spots are designated as A, B, C and D. (b) MPSE separated by either SDS-PAGE (1-D) or two-dimensional gel electrophoresis (2-D). Assay of 1:200 diluted serum from C57Bl/6 mice infected with *M. paratuberculosis*. Note the absence of the p24A spot shown in (a).

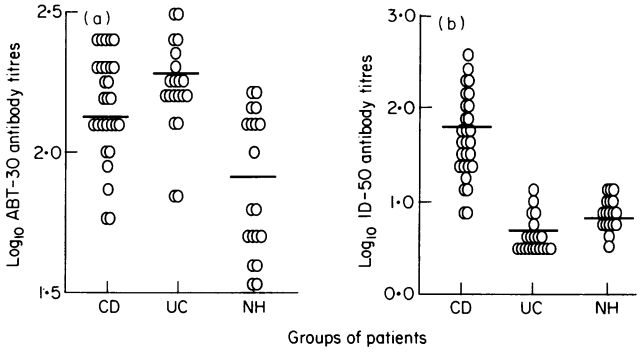

Fig. 4. Antibody levels to the 24-kD antigenic constituents in patients and controls. CD, Crohn's disease; UC, ulcerative colitis; NH, normal healthy individuals. Individual titres and group means (horizontal bars). (a) ELISA with the purified p24A antigen. (b) ELISA competition assay of antibodies to the 24-kD (BCD) antigens using the monospecific serum from *Mycobacterium paratuberculosis* (MAP)-infected C57Bl/6 mice.

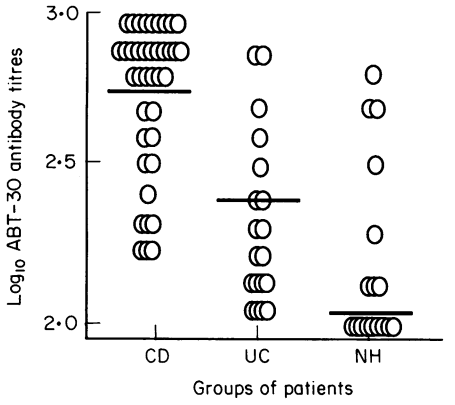

Fig. 5. Antibody levels to purified 18-kD protease-resistant antigen. ELISA assay of sera from patients and controls (see legend to Fig. 4).

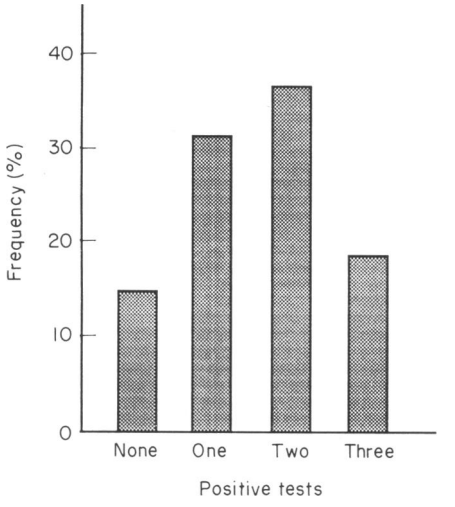

Fig. 6. Frequency of serological positivity in respect of the three antigens tested, p38, p24BCD and p18 antigens in patients with Crohn's disease.

24-kD band also in *M. kansasii* (results not shown) and also reacted in the two-dimensional Western blot with several other MAP antigens.

In contrast with the reaction of the goat serum, the pooled serum from MAP-infected C57Bl/6 mice reacted with the cathodic bands (B, C, D), but not with the anodic band (Fig. 3b). In view of these differences in specificity between the goat and mouse antisera it seemed necessary to investigate the reactions of sera from CD and UC patients with both the A and B, C, D 24-kD antigenic constituents.

The anodic 24-kD constituent (p24A) was eluted from polyacrylamide gel after two-dimensional electrophoresis. The protein eluted was used for the coating of microtitre plates for the titration of human sera by ELISA assay. The results showed large individual variations in both CD and UC patients' sera with mean $\log_{10}$ antibody titres $2{\cdot}1 \pm 0{\cdot}1$ and $2{\cdot}3 \pm 0{\cdot}2$ respectively (Fig. 4a). However, none of the patients had the antibody titre significantly ($3 \times$ s.d.) elevated above the mean titre ($1{\cdot}9 \pm 0{\cdot}2$) of the control group of healthy individuals.

It was not possible to carry out the ELISA testing of human sera in respect of the B,C,D constituents due to difficulties in purifying the heterogeneous cathodic 24-kD protein. Therefore, we employed the mouse serum pool which was found to be monospecific to the p24BCD constituents (see Fig. 3b) for the analysis of human sera by the ELISA competition assay. The results of the competition assay (Fig. 4b) showed that the $2{\cdot}0 \pm 1{\cdot}3$ mean $\log_{10}$ antibody titre of the CD patients was significantly ($P < 0{\cdot}001$) elevated above the $1{\cdot}0 \pm 0{\cdot}1$ mean titre of NH individuals and of the $0{\cdot}6 \pm 0{\cdot}2$ mean titre of UC patients. Thus, antibody levels in 53% of CD sera, but none of the UC sera, were found above the mean $+ 3 \times$ s.d. value of the NH control group.

Response to the 18-kD protease-resistant antigen

Interest in the protease-resistant bacterioferritin of MAP was stimulated by its species specificity and immunogenicity in ovine paratuberculosis [17] and in experimentally infected BALB/c mice [18]. The antigen was purified from protease-treated MPSE and used to test human sera by ELISA. The results represented in Fig. 5 showed the mean $\log_{10}$ antibody titre $2{\cdot}8 \pm 0{\cdot}2$ of the CD group significantly ($P < 0{\cdot}001$) increased above the mean titre $2{\cdot}0 \pm 0{\cdot}3$ of the NH group. In contrast, no significant difference was found in antibody levels in sera of UC patients with the mean titre of $2{\cdot}4 \pm 0{\cdot}2$ and the NH control group. Using the cut-off value of $+3 \times$ s.d. above the control NH group, elevated antibody levels were found in 53% of CD and in 10% of UC patients.

Lack of correlation between antibody specificities

When taking the serological results together, antibody levels in sera of CD patients were found to be elevated at the frequency of 57% in respect of the p38 antigen and of 53% in respect of the p24BCD and p18 antigens. Therefore, it was of interest to analyse whether the positivity for these serological assays overlapped in individual patients. This analysis was carried out by calculating the frequency of CD patients who were positive for any one, or any combination between the tested specificities (Fig. 6). The results showed that 18% of the patients were positive for all three tests, 14% of the patients were negative and the remaining patients were almost equally divided in having either one, or two positive tests. Hence, this analysis showed no significant correlation between any of the three examined antibody specificities. Finally, preliminary evaluation of antibody specificities in relation to disease activity in 11 patients has indicated that antibody positivity occurred more frequently in patients with diarrhoea, but without a preferential association of any of the three tested antibody specificities (results not shown).

DISCUSSION

In order to satisfy the requirements of specificity and sensitivity, the antigens were selected on the basis of prior analysis of the murine antibody repertoire, following infection with MAP and related strains of the *M. avium* complex [18]. Sera of CD patients containing antibodies to a 38-kD band of MAP have failed to react with a corresponding band of MTSE which is serologically the most prominent antigen in tuberculosis [19–21]. This protein presumably occurs in most bacterial species and has 30% sequence identity with the *Pho*S gene-coded phosphate-binding protein of *Escherichia coli* [24]. Despite the structural homology, studies in tuberculosis have suggested that antibodies to this

protein are directed against species-specific determinants [21]. Accordingly, at least one half of CD patients would have been sensitized either with MAP or with a sub-strain of *M. avium*, related to the isolate A6, which cross-reacted with the CD sera to a lesser degree. This finding however is surprising, when considering that the anti-38-kD antibody levels are elevated to a greater extent in multibacillary than in paucibacillary tuberculosis [21]. It is also paradoxical that anti-38-kD antibodies have not been detected in MAP-infected C57Bl/6 mice [18].

The two-dimensional gel analysis clearly demonstrated that antibodies from a goat with paratuberculosis reacted with one anodic (A) and several cathodic (B,C,D) 24-kD antigens, whereas serum from MAP-infected C57Bl/6 mice reacted only with the cathodic fractions. It was also interesting to find that sera from CD patients contained elevated antibody levels to the cathodic (B,C,D) fraction, representing the murine immunogen, whilst antibody levels to the p24A 'goat immunogen' were not significantly increased. It should be noted from the mouse infection model that p24BCD was MAP-specific on the basis of immunogenicity during MAP infection, whilst the binding of the resulting antisera was cross-reactive with homologous antigens from other species of *M. avium* [18]. These findings indicate that the species-specific epitopes of p24BCD are immunodominant and therefore may play a special role in the MAP-infected host. The striking species-specific immunogenicity can apparently discriminate between the various *M. avium* subspecies. In view of these special features, future gene cloning and sequencing of this protein could be of considerable interest.

The p18 antigen purified from *M. paratuberculosis* apparently corresponds to the recently characterized antigen 'D' [16]. This can be concluded on the basis of its resistance to treatment with proteinase K, and of corresponding molecular weight. Characterization of this protein by Brooks and colleagues revealed that the amino-terminal sequence has 50% sequence identity of this constituent with *E. coli* bacterioferritin, with a high content of leucine and glutamic acid and that the 18-kD subunits assemble in a native molecule of about 400 kD. On the basis of electron micrographs, this antigen is presumably contained in a electron-transmitting outer shell which is surrounding an inner electron-dense core. The presence of antibodies to the p18 antigen in sera of CD patients is also in accord with the recent report of positive serology in sheep affected by paratuberculosis [17]. The protease-resistance of antigenic determinants which are recognized by antibodies indicates an exceptional conformational stability of this protein, although its structural basis is not known. These properties, together with the peroxidase activity and possible role in the uptake, or storage of iron, may play a pathogenetic role in the infections with *M. paratuberculosis*.

Although antibodies to each of the three antigens tested were present in at least 50% of CD patients, it is important to note that their occurrence was not overlapping in individual patients and there was no significant correlation between the three antibody specificities tested. This finding has a number of implications for the overall interpretation of the serological data in respect of the possible etiological role of *M. paratuberculosis* in Crohn's disease. A conservative evaluation on the basis of serological positivity with all three antigens would suggest MAP infection in only 18% of CD patients. Alternatively, evaluation on the basis of serological positivity in respect of at least one of the antigens would implicate MAP infection in as many as 84% of patients. The accuracy of these figures is apparently limited by the cut-off points chosen for each test and by the relatively small number of patients examined. Nevertheless, it seems plausible to suggest that responsiveness of individual patients to some, but not all three, antigens tested can be attributed to genetic factors. This view is supported by the pronounced differences in antibody responsiveness to the p18 and p24BCD antigens between MAP-infected inbred strains of mice [18] and by the reported HLA-DR gene control of anti-p38 antibody levels in tuberculosis [25]. In addition to genetic factors, certain yet undefined differences between the infecting mycobacterial strains and considerable variation in the clinical and pathological features of CD could have contributed to the immunogenicity of different antigens. Despite these questions, which remain open, the results presented clearly support the role of *M. paratuberculosis* infection in the etiopathogenesis of Crohn's disease.

Note added in proof

It was demonstrated in our recent experiments that the anodic 24-kD (p24A) constituent corresponds to the cloned Av1-3 antigen reported by Yamaguchi *et al.* [15].

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