Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from patients with Crohn’s disease, ulcerative colitis and healthy controls: preliminary results

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ABSTRACT

Crohn’s disease (CD) is a gastro-intestinal inflammatory disease with uncertain origin. Autoimmune and/or infectious etiologies have been proposed as possible causes. A role for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in CD has been hypothesized. However, the role of MAP in causing this disease is subject to debate. The aim of the present study is to compare the rate of MAP isolation from patients living in an area of Northern Italy characterized by the presence of high prevalence of MAP infected cattle herds. In the region of concern, a mean annual incidence rate of 3.4 CD patients per 100,000 inhabitants has been reported. This study enrolled 186 patients: 23 patients with CD, 26 patients with ulcerative colitis (UC), 6 patients with not specific inflammatory bowel disease (IBD) and 131 healthy patients. For each patient, six biopsy tissue specimens from ileo-colon region were collected and processed for MAP isolation. Culture was performed with BacT/ALERT 3D system (bioMérieux) using a Middlebrook 7H9-based liquid medium. All samples were culture-negative for MAP after three months of incubation. An acid-fast, IS900 negative organism was recovered from a healthy control patient. The isolate was able to grow in the absence of mycobactin J. At present, our findings do not support a role for MAP as a microbial causal agent of CD. A larger number of CD patients and an extension of incubation times of cultures are needed to make clear the pathogenic relationship between MAP and CD in this region.

Key words: Crohn’s disease, biopsies, culture, paratuberculosis, PCR.

INTRODUCTION

Crohn’s disease (CD) is a human gastro-intestinal inflammatory disease of uncertain origin. A role for *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causal agent of paratuberculosis in ruminants, in CD was first considered following the isolation of three strains of a MAP-like organism from 3 of 14 patients with CD (Chiodini et al., 1984). However, the involvement of MAP in the etiology of CD is still under debate. Main evidence supporting the causal link are: (a) many studies (Quirke, 2001; Grant, 2005) have indicated a higher presence of MAP in patients with CD than in controls either by culture and/or by PCR or hybridization methods using biopsies and/or on surgically resected tissue samples; (b) a therapy based on broad spectrum antibiotics believed effective against MAP such as rifabutin, clarithromycin and clofazimine, led to good results in the treatment of CD (Borody et al., 2002; Shafran et al., 2002). Moreover, the presence of MAP has been detected in blood samples from Crohn’s patients (Naser et al., 2004). Nevertheless, other authors have reported the absence of a significant positive correlation between CD and the frequency of detection of MAP (Chiba et al., 1998; Kanazawa et al., 1999; Van Kruiningen, 1999).

Sceptics do not accept the hypothesis of an active role of MAP in CD. There is insufficient evidence to describe the effect of antibiotic treatments against MAP specifically rather than against other enteric bacteria. The fact that CD patients’ symptoms do not worsen when treated with immunosuppressive agents is not in keeping with an infectious cause of Crohns disease. Patients whose tissues are reported to contain MAP in an obligate intracellular spheroplast form do not present a cell mediated immune response to MAP antigens. Finally, there is no evidence that humans living in contact with animals infected with MAP have a higher risk of developing the disease for (Sartor, 2005). Many infectious agents other than MAP have been suggested as causes of CD, including the measles virus, *Listeria* spp. and *Escherichia coli* (Grant, 2005). A possible commensal presence of MAP in CD patients has been also hypothesized. On the other hand, a mutation in the gene encoding nucleotide-binding oligomerization domain 2 (NOD2) has
been associated with a susceptibility to CD (Hugot et al., 2001; Ogura et al., 2001). Moreover, functional variants of organic cation transporter (OCTN) genes are associated with CD (Peltekova et al., 2004). Taken together, these findings indicate a complex polygenic nature of the disease.

A contentious debate is in progress as indicated by criticisms addressed to some of the studies proposing that MAP might be a cause of CD (Roholl et al., 2002; Gaya et al., 2004; Huggett et al., 2004). The picture shared by most scientists is that environmental risk factors add to predisposing gene variations in leading to an abnormal immune response at the intestinal level.

The aim of the study was to compare the rate of MAP isolation from human patients living in an area of Northern Italy characterized by a high prevalence of MAP-infected cattle herds.

MATERIALS AND METHODS

Population and study area
The study was carried out on 186 subjects presenting to the referral hospitals of the Provinces of Crema (Ospedale Maggiore) and Cremona (Ospedale Maggiore), Region Lombardia, Northern Italy, with symptoms compatible with an inflammatory bowel disease (IBD). Patients underwent a diagnostic work-up and were assigned to four different groups: patients with CD, patients with ulcerative colitis (UC), patients with non-specific IBD and healthy patients.

Diagnostic criteria
For each patient, clinical information was carefully considered. The diagnosis of IBD was made on the basis of clinical, endoscopic, radiological and histological criteria. In some patients the diagnosis was further confirmed by serological determination of anti-Saccharomyces cerevisiae antibodies (ASCA), of anti-neutrophil cytoplasmic antibodies (p-ANCA) and by videocapsule endoscopy. ASCA+pANCA (-) results are mostly associated with CD patients, whereas ASCA-pANCA (+) results are mostly associated with UC patients or with patients with an UC-like form of CD. Pathological findings mimicking IBD and with an alternative diagnosis were excluded.

Samples
At present, the study enrolled 186 patients: 23 patients with CD, 26 patients with UC, 6 patients with non-specific IBD and 131 healthy patients. For each patient, six tissue specimens from the ileo-colon region were collected and processed for MAP isolation.

Culture
The six biopsy tissue specimens were collected from the patient and immediately homogenized in 5 ml of sterile saline. The suspension was diluted to 50 ml with sterile saline and 5 ml of digestion reagent was added (0.75 N NaOH, 0.114 M sodium citrate tribasic – Biolife Italiana, Milano, Italy). After agitation the suspension was incubated at 37°C for 5 min and than for 10 min at room temperature (RT) with continuous agitation. The suspension was then added to 40 ml of phosphate buffer (0.032 M potassium phosphate monobasic, 0.031 M potassium phosphate dibasic, pH 6.8 - Biolife Italiana, Milano, Italy), mixed and centrifuged at 3000 × g for 20 min at 10°C. The pellet was resuspended with 0.5 ml of phosphate buffer and inoculated in a Middlebrook 7H9-based liquid medium, namely BacT/ALERT MP Process Bottle (bioMérieux, Durham, North Carolina), plus MB/BacT Antibiotic Supplement, BacT/ALERT MB Enrichment Fluid and 0.0002% (wt/vol) mycobactin J (Allied Monitor, Fayette, MO). The samples were incubated at 35-37°C in the BacT/ALERT 3D system for times ranging from 42 to 90 days and monitored for microbial growth. The suitability of the BacT/ALERT 3D system to support MAP growth was assessed by cultivation of the ATCC 19698 strain.

Identification of MAP
The protocol provides that culture-positive samples are identified as MAP-positive on the basis of Ziehl-Neelsen staining and confirmed on the basis of the mycobactin J-dependence of the bacterium and by IS900-PCR.
DNA extraction

DNA extraction was performed on isolated acid-fast organisms by a phenol/chloroform method. Briefly, 100 µl of cultured bacterial suspension were pelleted, resuspended with 100 µl of TE buffer (1 mM tris/HCl, pH 7.6, 0.1 mM EDTA) and added with 100 µl of phenol/chloroform. The suspension was mixed by inversion of the tube and centrifuged. One-hundred µl of the aqueous phase were recovered and transferred to a clean tube. After extraction, the DNA was precipitated at RT with one volume of isopropanol and one-tenth volume of 3 M NaOAc (pH = 4.8). The pellet was then washed once with 70% ethanol, resuspended with 20 µl of deionized sterile water and stored at –20°C until amplification.

PCR

The PCR was performed by using IS900-specific primers, namely P90 – P91 (Schwartz et al., 2000) either on the extracted DNA or directly on the cultured bacterial suspension which had been previously washed and resuspended with sterile PBS. The reactions were carried out in 50 µl containing 2 U of Taq DNA polymerase (Klen Taq 1, Ab Peptides, Inc – USA), 1X PCR buffer (1X PC2), 0.2 mM of each dNTP, 0.5 µM of each primer, and 5.0 µl of DNA sample. PCR was performed by 35 amplification cycles, each consisting of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and chain elongation at 72°C for 1 min. In the first cycle the samples were denatured at 95°C for 5 min and then rapidly ice-cooled before polymerase addition. In the last cycle the extension step was increased to 10 min. Detection of amplified products was performed on 2% agarose gel by ethidium bromide staining.

RESULTS

All biopsy tissues from all four diagnostic categories were culture negative for MAP. An acid-fast organism other than MAP was recovered from a healthy control patient. The isolate was negative when tested by IS900-PCR and was able to grow in the absence of mycobactin J, although less robustly than in media containing this iron chelating factor.

DISCUSSION

In the study area, a mean annual incidence rate of 3.4 CD patients and of 7.0 UC patients per 100,000 inhabitants has been reported by a study based on a four year observation period (Ranzi et al., 1996). These values are relatively high when compared to data regarding other Italian areas (Tragnone et al., 1996). Moreover, this area of Northern Italy is characterized by a high prevalence of MAP-infected cattle herds. However, no studies have been made to detect a correlation between a high prevalence of CD and a high prevalence of MAP-infected cattle herds. A possible route of MAP transmission of MAP from infected cattle to human beings may be milk, since MAP can be present in milk due to direct secretion and/or fecal contamination. Pasteurization is able to reduce the degree of milk contamination, but does not guarantee a complete elimination of MAP (Grant, 2005). Less data are reported about the persistence of MAP in milk products. Studies regarding the survival of MAP in cheeses, carried out using experimentally MAP-contaminated milk, showed an incomplete inactivation of MAP in cheddar, semihard and hard cheeses during manufacturing and ripening (Spahr and Schafroth, 2001; Donaghy et al., 2004).

Anamnestic data of the examined patients has not been considered in this phase of the study. Interesting data could also be derived from the serological analysis of collected sera from CD patients included in the study.

The difficulty in isolating MAP from tissue samples of human origin has to be taken into account in interpreting these results. One of the difficulties for the isolation of the micro-organism is the possible presence of the bacterium as wall-deficient spheroplast form in tissues. Moreover, in this study, biopsies were utilized due to the unavailability of surgically resected tissue samples which are quantitatively and qualitatively more suitable for isolation of MAP (Schwartz et al., 2000). However, positive cultures for MAP from human microscopic biopsies have been reported by using the Becton-Dickinson mycobacterial growth
indicator tube (MGIT) system (Schwartz et al., 2000). Other attempts to grow MAP from human intestinal mucosal biopsies on MGIT have also been made (Bull et al., 2003). In the present study, multiple tissue samplings from each patient were made in order to increase the sensitivity of the test. In this way a greater amount of tissue from different mucosal areas was collected. However, this study’s sampling technique does not reach the deeper layer of the mucosa and this limitation could affect the ability to isolate MAP, as hypothesized by Schwartz and co-workers (2000).

The suitability of the BacT/ALERT 3D system to support MAP growth was assessed by cultivation of the ATCC 19698 strain. Success in MAP isolation differs depending on the culture systems that used. An example is reported by Schwartz and co-workers (2000) who found MAP-positive MGIT cultures but MAP-negative BACTEC cultures in primary isolation of MAP from the same clinical specimens. Both systems, and the BacT/ALERT 3D system as well, use media deriving from Middlebrook 7H9-broth base. Different rates of MAP isolation could be due to the presence of different media additives in the two systems. Finally, another parameter to be considered is the incubation time. At present, for this study, cultures have been incubated for 3 months and longer incubation times are needed to exclude false-negative results.

CONCLUSION

In this preliminary study, MAP was not isolated from multiple intestinal mucosal biopsies collected from patients with CD, patients with UC, with non-specific IBD and from healthy patients. At present, our findings do not support a role for MAP as microbial causal agent of CD. The samples are still incubating after three months and the study is still in progress. Either a higher number of CD-affected patients or an extension of incubation times of cultures are needed to make clear the pathogenetic relationship between MAP and CD in this region.

REFERENCES


