P-ANCA in monozygotic twins with inflammatory bowel disease

P Yang, G Järnerot, D Danielsson, C Tysk, E Lindberg

Abstract
Perinuclear antineutrophil cytoplasmic antibodies (P-ANCA) have been demonstrated in patients with ulcerative colitis and in a higher frequency than expected in their first degree relatives. A hypothesis was proposed that P-ANCA is genetically determined and may represent a subclinical marker of genetic susceptibility to ulcerative colitis. This study analysed P-ANCA in monozygotic twins with inflammatory bowel disease to evaluate this hypothesis further. P-ANCA was analysed with indirect immunofluorescence technique in 12 monozygotic twin pairs with ulcerative colitis and 14 twin pairs with Crohn’s disease. Furthermore, the study included 21 non-twin patients with ulcerative colitis, 18 non-twin patients with Crohn’s disease, and 52 healthy controls matched for sex and age. In ulcerative colitis P-ANCA occurred in nine of 14 (64.3%) monozygotic twins and in 13 of 21 (61.9%) non-twin cases, which was significantly different compared with healthy controls who were positive in three of 52 (5.8%) cases (p < 0.0001). P-ANCA was found in two of 10 (20%) healthy twin siblings to twins with ulcerative colitis, which was not significantly different from healthy controls (p = 0.18). The results in Crohn’s disease did not differ from healthy controls. Previous findings of P-ANCA occurring in ulcerative colitis but not in Crohn’s disease are supported. This study does not support the hypothesis that P-ANCA is a subclinical marker of genetic susceptibility to ulcerative colitis.

Methods

Twins
Our studied twin population has been described in detail.3-11 Thirty-four unselected monozygotic twin pairs with inflammatory bowel disease (IBD) were found in that study. Those who were younger than 75 years of age and with both twins in each pair still alive were invited to participate in the investigation. Two recently diagnosed monozygotic twin pairs with IBD were also invited. Fifty-two of 66 subjects agreed to participate constituting 14 pairs with Crohn’s disease and 12 pairs with UC. Five pairs with Crohn’s disease and two pairs with UC were concordant for the disease. The study was approved by the ethical committee, Örebro Medical Center Hospital.

Non-twin IBD cases
Thirty-nine patients with IBD (21 UC, 18 Crohn’s disease) attending our colitis clinic were tested for the occurrence of P-ANCA. Eleven of the UC patients were considered to have active disease estimated from conventional clinical and endoscopic criteria while 10 were inactive. In the Crohn’s disease group five were active and 13 inactive. None of these patients were treated with corticosteroids or azathioprine at the time of the study.

Healthy controls
One healthy control subject for each twin was chosen from members of the staff or blood
donors, all without any history of gastrointestinal disease. In total, 52 subjects were matched for sex and age within two years.

**P-ANCA**

P-ANCA was detected in ethanol fixed neutrophils by indirect immunofluorescence. Briefly, heparinised venous blood was drawn from two healthy members of the staff and neutrophils were separated by Ficoll-Paque (Kabi-Pharmacia, Uppsala, Sweden) density centrifugation according to the method of Boyum. Glass slides with neutrophils on a well defined area were prepared by means of cytocentrifugation (Shandon Cytospin, Cheshire, England), fixed in fresh 99% ethanol for 5 to 10 minutes at +4°C, air dried, and stored in the refrigerator until use. Serum samples from patients and healthy controls were diluted 1:10 in phosphate buffered saline, pH 7-4; and then tested for the presence of P-ANCA. Approximately 33 μl of the diluted serum was applied on the well defined area of the glass slide with neutrophils and incubated in a humid chamber for 30 minutes at room temperature. After washing with phosphate buffered saline for 10 minutes and incubation for another 30 minutes with a 1:10 dilution of FITC labelled F(ab)2 rabbit antihuman IgG antibodies (Dakopatts A/S, Copenhagen, Denmark), the slides were washed again in phosphate buffered saline for 10 minutes and finally mounted under a cover slip with phosphate buffered saline-glycerin. The slides were scrutinised at a magnification of 250X or 420X under a Zeiss epifluorescence microscope equipped with a 50 W tungsten lamp, BD 450-490 as primary filter and LP520 as barrier filter. Serum samples giving a typical perinuclear staining reaction with the neutrophils were regarded as positive. They were always retested at the initial dilution of 1:10 and further at twofold dilutions of 1:20-1:640. All samples were analysed blindly, and the code was broken when all the analyses had been performed.

**Statistics**

Fisher’s exact test and McNemar test were used for statistical analysis, and a p value <0.05 was considered statistically significant.

**Results**

**Patients**

In twins with UC the mean age at diagnosis was 27.7 years (17-45) and the actual mean age was 49.1 years (24-74). All were in clinical remission with normal concentrations of haemoglobin, C reactive protein, and serum orosomucoid. Four patients had an ileostomy. In the others the rectal mucosa was macro and microscopically inactive.

The mean age at diagnosis in twins with Crohn’s disease was 28.5 years (20-45), and the actual mean age was 42.9 years (34-63). Two twins had mild diarrhoea and slightly increased C reactive protein and orosomucoid concentrations. They were treated with sulphasalazine. The remaining twins were in remission. Three of them had an ileostomy.

A thorough interview did not reveal symptoms suggestive of IBD in the healthy twins. They had remained healthy for an average of 21.4 years (8-40) after diagnosis in the twins with UC and 14.9 years (7-31) in the twins with Crohn’s disease. All had a normal rectal mucosa and histopathological examinations were normal as well.

In the non-twin IBD group the UC patients had an mean age of 44 (21-73) years and the Crohn’s disease group 47 (20-81) years.

**P-ANCA**

Positive P-ANCA refers to a perinuclear staining pattern whereas no case of cytoplasmatic staining was found.

Nine of 14 (63.6%) identical twins with UC had a positive P-ANCA and 13 of 21 (61.9%) non-twin UC cases. Of the 10 healthy monozygotic twins two had P-ANCA (20%). In one of these two twin pairs both the diseased and the healthy twin were P-ANCA positive. In the other pair the healthy twin had a high P-ANCA titre (1/640) while the diseased twin was negative. In the two pairs concordant for UC both twins in one pair were P-ANCA positive while in the other pair one was positive and the other P-ANCA negative (Table I).

One of 19 (5.3%) of identical twins with Crohn’s disease had a positive P-ANCA and one of 18 (5.6%) non-twin CD cases. Of the nine healthy monozygotic twins none had a positive P-ANCA. Of the healthy control subjects three of 52 (5.8%) had a positive P-ANCA. Table II summarises the results.

<table>
<thead>
<tr>
<th>Twin pair number</th>
<th>Distribution of disease</th>
<th>Titer of P-ANCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UC H</td>
<td>0/0</td>
</tr>
<tr>
<td>2</td>
<td>UC H</td>
<td>1/10</td>
</tr>
<tr>
<td>3</td>
<td>UC H</td>
<td>0/0</td>
</tr>
<tr>
<td>4</td>
<td>UC H</td>
<td>0/0</td>
</tr>
<tr>
<td>5</td>
<td>UC H</td>
<td>0/160</td>
</tr>
<tr>
<td>6</td>
<td>UC H</td>
<td>0/0</td>
</tr>
<tr>
<td>7</td>
<td>UC H</td>
<td>0/1/40</td>
</tr>
<tr>
<td>8</td>
<td>UC H</td>
<td>1/10</td>
</tr>
<tr>
<td>9</td>
<td>UC H</td>
<td>1/1/20</td>
</tr>
<tr>
<td>10</td>
<td>UC H</td>
<td>0/0</td>
</tr>
<tr>
<td>11</td>
<td>UC H</td>
<td>1/10</td>
</tr>
<tr>
<td>12</td>
<td>UC H</td>
<td>1/10</td>
</tr>
</tbody>
</table>

UC=twin with ulcerative colitis; H=healthy twin sibling.

**Table I: Distribution and results of P-ANCA in monozygotic twins concordant or discordant for ulcerative colitis**

**Table II: Distribution of P-ANCA in all monozygotic twins with ulcerative colitis (UCw) and their healthy twin siblings (H-UCw), in monozygotic twins with Crohn’s disease (CDw) and their healthy twin siblings (H-CDw), in non-twin cases with ulcerative colitis (UC) and Crohn’s disease (CD), and in healthy controls**

<table>
<thead>
<tr>
<th>Control</th>
<th>P-ANCA+</th>
<th>P-ANCA−</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCw</td>
<td>9</td>
<td>5</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>UCw</td>
<td>1</td>
<td>8</td>
<td>p=0.18</td>
</tr>
<tr>
<td>CDw</td>
<td>1</td>
<td>18</td>
<td>p=0.71</td>
</tr>
<tr>
<td>H-UCw</td>
<td>0</td>
<td>9</td>
<td>p=0.61</td>
</tr>
<tr>
<td>UCw</td>
<td>13</td>
<td>8</td>
<td>p=0.0001</td>
</tr>
<tr>
<td>CDw</td>
<td>1</td>
<td>17</td>
<td>p=0.73</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>5</td>
<td>49</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Fisher’s exact test versus healthy controls; ND=not done.*
The prevalence of P-ANCA was significantly higher both in the monozygotic UC twins and in the non-twin UC cases compared with healthy control subjects (p<0.0001). The frequency of P-ANCA in the monozygotic twins with UC was significantly higher than in the corresponding healthy twin siblings (p=0.047; Fisher's exact test). The distribution of P-ANCA within the 10 twin pairs discordant for UC showed a difference which, however, did not reach statistical significance (p=0.11; McNemar test) (Table III). Obviously, this is an effect of the limited number of cases in this analysis. In the Crohn's disease groups no statistical differences occurred between twins, non-twin cases, and healthy controls. The occurrence of P-ANCA was not influenced by disease activity (not shown).

Discussion
The aetiology of UC is unknown, probably multifactorial, and dependent on the interplay of genetic and environmental factors. Complex segregation analysis has shown the presence of a major dominant/additive gene with a penetrance of 0.20–0.26 in about 10% of the patients with UC. This means that the genotype is normal in about 90% of adult UC patients. The concordance rate in the twins with UC was one of 16, which is compatible with the results in the segregation analysis. In addition to P-ANCA, several subclinical markers have been suggested to aid genetic studies in IBD. However, studies of HLA-antigens, complement allootypes, allo-types of immunoglobulins, T cell receptor chains, lymphocytotoxic antibodies, antibodies to colonic epithelial cells, and intestinal permeability have not shown any consistent patterns. The analysis of colonic glycoproteins in monozygotic twins showed a genetically defined change, which occurred both in healthy twins and twins with UC.

In UC, the antigens to which P-ANCA reacts are not fully identified, but the antibody has probably no pathophysiological importance. It is generally accepted that there is no correlation between P-ANCA and the extent, duration or clinical activity of UC and the antibody generally persists after proctocolectomy. P-ANCA may rather represent a marker of an underlying immunoregulatory disturbance. An association between HLA-DR2 and P-ANCA positive UC was reported whereas an increased frequency of HLA-DR4 was seen in P-ANCA negative UC. The increased frequency of P-ANCA reported in healthy first degree relatives to probands with UC raised the hypothesis that P-ANCA may be genetically determined. Furthermore, the possibility of genetic heterogeneity was suggested as the prevalence of P-ANCA in relatives to P-ANCA positive probands was significantly higher than in P-ANCA negative probands. If genetics are of fundamental importance for the occurrence of P-ANCA, a much higher frequency of this antibody would be expected in healthy monozygotic twins than in patients with UC compared with other first degree relatives. This was not the case in our study with twins.

We confirm previous results of P-ANCA in patients with IBD. Twenty two of all 35 UC cases (62.9%) were P-ANCA positive and only two of all 37 cases with CD (5.4%). The sensitivity of P-ANCA is within the limits in previous reports. Our data of twin siblings, however, do not support the hypothesis that P-ANCA might be a genetic marker in UC. This conclusion is mainly based on the findings in the healthy twin siblings to twins with manifest UC. Only two of 10 healthy twin siblings (20%) were P-ANCA positive, which was not significantly different from healthy controls (p=0.18; Fisher’s exact test). The frequency of P-ANCA in the monozygotic twins with UC was significantly higher than in the corresponding healthy twin siblings (p=0.047; Fisher’s exact test). The distribution of P-ANCA within the 10 twin pairs discordant for UC showed a difference which, however, did not reach statistical significance (p=0.11; McNemar test). Obviously, this is an effect of the limited number of cases in this analysis. All performed statistical tests taken together, however, give no support for a major genetic influence on the expression of P-ANCA and the results of this study do not favour P-ANCA as a marker of genetic susceptibility of UC.

The discrepancies between our findings and those by Shanahan’s group and Seibold et al. could be caused by the different types of relatives studied as we restricted our study to monozygotic twins. However, there might also be methodological differences. The whole cell enzyme linked immunosorbent assay (ELISA) used by Shanahan et al. might detect anti-neutrophil cytoplasmic antibodies other than those responsible for P-ANCA, which is based on morphological criteria. Although the whole cell ELISA and indirect immunofluorescence showed ‘excellent agreement’ the higher sensitivity, however, of the ELISA technique has not yet been confirmed by others.

In summary, the frequency of P-ANCA was not significantly increased in healthy monozygotic twin siblings to twins with ulcerative colitis compared with healthy controls. Our data are in accordance with the French, British, and Italian studies but in contrast with the American and German findings. Whether ethical factors influence the results is unknown and cannot be stated from our data.

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