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Lack of effect of antineutrophil cytoplasmic antibodies associated with ulcerative colitis on superoxide anion production from neutrophils

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Abstract

***Background*—Antineutrophil cytoplasmic antibodies (ANCAs) from patients with vasculitidis can induce neutrophils to release oxygen radicals in vitro. ANCAs with a perinuclear pattern of immunofluorescence are found in most patients with ulcerative colitis, but several findings are against ANCAs having a pathogenetic role in this disease.**

***Aims*—To evaluate the influence of ANCAs associated with ulcerative colitis on the respiratory burst activity of neutrophils.**

***Patients*—Serum samples were obtained from 14 patients with ulcerative colitis, seven of whom showed positivity for p-ANCAs, three patients with vasculitidis, two with positivity for p-ANCAs, and one for c-ANCAs, and seven healthy volunteers.**

***Methods*—A positive ANCA serology was determined with a standard indirect immunofluroescence assay. Purified immunoglobulins (IgGs) were prepared from serum samples by DEAE-Affigel blue chromatography. Human neutrophils were prepared by dextran-Ficoll-Hypaque separation. Superoxide anion ($O_{2^-}^{\cdot}$) generation was measured by following the superoxide dismutase inhibitable reduction of ferricytochrome.**

***Results*—There were no significant differences among samples from ulcerative colitis IgG p-ANCA positive, ulcerative colitis IgG p-ANCA negative patients, and controls on $O_{2^-}^{\cdot}$ production, whereas ANCA positive IgG from vasculitidis significantly enhanced $O_{2^-}^{\cdot}$ release ($p<0{\cdot}001$).**

***Conclusions*—p-ANCAs associated with ulcerative colitis have no effect on the respiratory burst activity of normal human neutrophils in vitro. These results reinforce the hypotheses that ANCAs are unlikely to contribute to the pathogenesis of ulcerative colitis.**

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Keywords: antineutrophil cytoplasmic antibodies, ulcerative colitis, respiratory burst, superoxide anion.

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Antineutrophil cytoplasmic autoantibodies (ANCAs) are antibodies that react with antigens within the cytoplasm of neutrophils.

The association of ANCAs with inflammatory vascular diseases, such as Wegener's granulomatosis, microscopic polyarteritis, and crescentic glomerulonephritis has long been recognised.[1 2] These antibodies have proved useful as serological diagnostic markers and indicators of disease activity.[3] Furthermore, a possible role for ANCAs in the pathogenetic mechanisms of these diseases, via neutrophil activation, has been strongly suggested; ANCA-positive serum samples from patients with necrotising vasculitis stimulate neutrophils to degranulate, to produce reactive oxygen radicals, and to damage endothelial cells in vitro.[4–7]

Recently, the presence of ANCAs with a perinuclear pattern of immunofluorescence has been shown in association with ulcerative colitis.[8–13] By contrast with what is suggested in vasculitic disorders,[14] several findings are against ANCAs having a pathogenetic role in ulcerative colitis[15]; however, whether p-ANCAs associated with ulcerative colitis have a functional role in neutrophil activation is still unknown. The aim of the present study was to determine whether p-ANCAs from patients with ulcerative colitis have an effect on the respiratory burst activity of normal human neutrophils in vitro.

Methods

SERUM AND IMMUNOGLOBULIN SAMPLES

Serum samples were obtained from 24 subjects – 14 had ulcerative colitis (seven p-ANCA positive and seven p-ANCA negative), three had vasculitic disorders (two with polyarteritis, p-ANCA positive with antimyeloperoxidase activity and one with Wegener's granulomatosis, c-ANCA positive and with anti-proteinase 3 activity), and seven were healthy volunteers (all ANCA negative). A positive ANCA serology had been determined by a standard indirect immunofluorescence assay as described previously.[9 12] Briefly, slides containing cytocentrifuged human neutrophils from a normal healthy donor were washed in phosphate buffered saline (PBS), pH 7·2, for 30 minutes. Slides were then incubated with normal rabbit serum diluted 1:50 in PBS for 15 minutes, and subsequently with human serum diluted 1:20 in PBS for 30 minutes. After further washing in PBS, slides were incubated with fluorescein-conjugated rabbit F(ab')2 antihuman IgG (Southern Biotechnology Associates Inc, Birmingham, AL, USA) for 30 minutes. All incubations were performed at room temperature in a humid chamber. Slides were then thoroughly washed

in PBS, mounted in Elvanol, and examined for immunofluorescence reactivity by two independent observers with a microscope equipped with epifluorescence. Samples showing the typical patterns were considered positive for c-ANCA or p-ANCA. To avoid any possible effect of serum contaminants on activation of neutrophils, serum IgGs were prepared from all serum samples by DEAE-Affigel blue chromatography (Bio-Rad Laboratories BV Veenendaal, The Netherlands). The purity of these preparations was checked by SDS-polyacrylamide electrophoresis. The resulting IgGs were then dialysed overnight against PBS, pH 7·4, which was the buffer subsequently used for the neutrophilic activation assay. Preparations of IgGs were kept at −20°C until the time of the assay.

ISOLATION OF NEUTROPHILS

The study was performed with neutrophils from one normal subject. They were prepared from 30 ml heparinised peripheral blood by a combined dextran-Ficoll-Hypaque separation procedure. Neutrophils were mixed with 3 ml 6% dextran and allowed to stand at 37°C for 30 minutes; the leucocyte rich plasma was layered over an equal volume of Ficoll-Hypaque gradient (Pharmacia, Uppsala; Sweden) and centrifuged at 400 *g* for 30 minutes. Contaminating erythrocytes were removed by hypotonic lysis by resuspending the cells in triammonium chloride solution for 10 minutes. The resulting cells (>95% neutrophils) were washed twice in PBS (125 nM NaCl, 8 mM NaHPO; 2 mM KCl, 5 mM glucose) at pH 7·4 and then resuspended in PBS and stored on ice until use. The viability of neutrophils, as determined by tryphan blue exclusion, was more than 96%.

SUPEROXIDE GENERATION ASSAY

Superoxide radical ($O_2^{\cdot-}$) production by neutrophils was measured by following the superoxide dismutase inhibitable reduction of ferricytochrome c (cyt-c) at 550 nm in a dual beam spectrophotometer.

For each experiment $1{\cdot}5\times10^6$ cells, suspended in PBS, were incubated with IgG at 100 μg/ml concentration for five minutes at 37°C before the reaction was initiated by the addition of 0·1 μg/ml of phorbol myristate acetate (PMA). Release of $O_2^{\cdot-}$ was calculated from the linear portion of the cyt-c reduction plot using a molar absorption coefficient of $21{\cdot}1\times10^3$/M/cm.

Proteins were determined according to Bradford using bovine serum albumin as a standard.[16]

Experiments were done in triplicate on separate aliquots of cells.

STATISTICAL ANALYSIS

Results are expressed as median and range and statistical comparisons were performed by Mann-Whitney *U* test.

Results

Preliminary measurements showed that in the absence of PMA, isolated neutrophils did not produce spontaneous $O_2^{\cdot-}$ formation.

Figure 1 shows the results of superoxide release after PMA stimulation of neutrophils. In a continuous assay there were no significant differences in $O_2^{\cdot-}$ production, between p-ANCA positive, and p-ANCA negative IgGs from patients with ulcerative colitis and IgGs from normal controls (28·4 (20·9–37·5); 26·6 (19·9–40·4); 31·3 (16·5–42·0) μmol/min/mg protein respectively); by contrast incubation of neutrophils with ANCA positive IgGs from patients with vasculitis resulted in a significantly greater superoxide release than ANCAs from patients with ulcerative colitis and controls (61·7 (57·4–65·2) μmol/min/mg protein ($p<0{\cdot}001$)). Figure 2 shows the comparative kinetics of $O_2^{\cdot-}$ release. After stimulation with PMA there is an immediate increase in $O_2^{\cdot-}$ ion, significantly greater from neutrophils incubated with ANCA positive IgG derived from patients with vasculitis.

Discussion

Our data indicate that p-ANCA positive or p-ANCA negative IgGs from patients with ulcerative colitis did not differ from IgGs from controls in their effects on respiratory burst

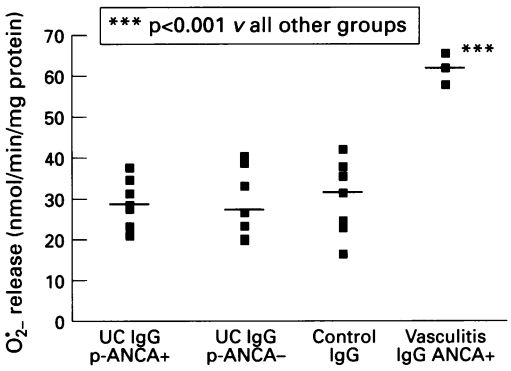

*Figure 1: Superoxide anion production measured in a continuous assay by superoxide dismutase inhibitable reduction of ferricytochrome c. UC=Ulcerative colitis. ***p<0·001 v all other groups.*

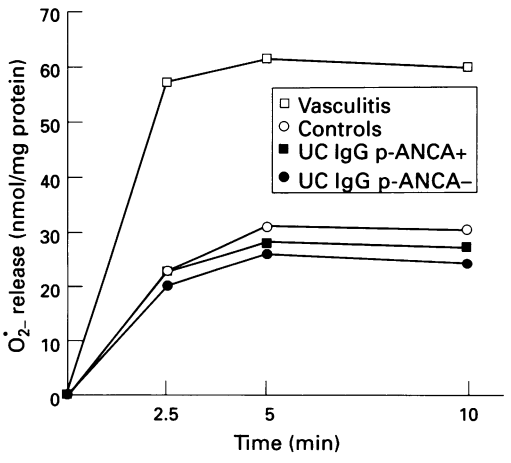

Figure 2: Comparative kinetics of superoxide release from neutrophils incubated with various IgGs after stimulation with 0·1 μg/ml PMA. UC=Ulcerative colitis.

activity of normal human neutrophils in vitro. By contrast, ANCA positive IgGs from patients with vasculitic disorders significantly enhanced O_2^- release, confirming previous findings by others.[5] In vasculitic disorders the antigens reactive with circulating IgG have been identified; in particular, Wegener's granulomatosis is characterised by a cytoplasmic reactivity of ANCA, and the antigen is proteinase 3.[17-19] Other vasculitic conditions, such as polyarteritis or crescentic glomerulonephritis, are characterised by the presence of perinuclear ANCA and the antigen is myeloperoxidase.[20] In patients with ulcerative colitis, whose serum samples react with neutrophils with a distinct perinuclear pattern, the specific antigen is still unknown. Only a few patients with ulcerative colitis had serum samples with positive p-ANCA immunofluorescence that reacted with known neutrophilic antigens, and the antigens are different in different patients.[13]

The fact that in the present study ANCA IgGs from ulcerative colitis did not stimulate neutrophilic activation is further evidence of the diversity of ulcerative colitis specific antigen compared with vasculitis.

Several data indicate that p-ANCAs do not exert a critical pathogenetic role in ulcerative colitis. Firstly, the prevalence and titre of ANCAs do not correlate with disease activity or extent.[9 12 21] Also, 20%–50% of patients with ulcerative colitis are ANCA negative and, on the other hand, ANCAs can occur in unaffected relatives.[22 23]

On the basis of these data, ANCAs are suggested as immunological markers of disease heterogeneity and disease susceptibility rather than pathogenetic mediators.

Recently, it has been noted that colonic mucosa is the specific and unique site of p-ANCA production in patients with ulcerative colitis and that serum p-ANCA may represent a spill over of the locally produced antibodies.[25] Therefore p-ANCAs may still exert a pathogenetic role in ulcerative colitis by means of local pathogenetic mechanisms, such as activation of neutrophils. Our study, however, by showing that ANCAs associated with ulcerative colitis do not influence the respiratory burst of normal human neutrophils in vitro, is strong evidence against this hypothesis.

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