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Anti-endothelial cell antibodies in patients with rheumatoid arthritis complicated by vasculitis

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SUMMARY

IgG antibodies reactive with human umbilical vein endothelial cells were found in 19 out of 28 patients with rheumatoid vasculitis (RV), in four out of 24 patients with rheumatoid arthritis (RA), in seven out of 10 patients with systemic lupus erythematosus (SLE), but not in healthy donors. In four patients with RV who were followed longitudinally, regression of vasculitic episodes coincided with decreasing titres of anti-endothelial antibodies (AEA). Binding activity to endothelial cells was observed in intact IgG and in $F(ab')_2$ fragments of IgG. AEA activity was unrelated to antibodies against nuclear, blood group or major histocompatibility complex antigens and did not involve immune complexes. AEA activity was not specific for endothelial cells since the AEA-positive sera and the IgG fractions prepared from these sera also reacted with fibroblasts. Adsorption of positive sera and corresponding IgG fractions with endothelial cells decreased the IgG binding reactivity on both fibroblasts and endothelial cells. These findings show that RV patients have IgG–AEA, and suggest that these antibodies may play a role in the pathogenesis of the disease.

Keywords endothelium rheumatoid vasculitis

INTRODUCTION

Vascular inflammation is inherent in the pathogenesis of several extra-articular manifestations of rheumatoid arthritis (RA). Skin rash, cutaneous ulcerations, gangrene, neuropathy and abnormalities of visceral organs have been described in association with rheumatoid vasculitis (RV) (Scott, Bacon & Tribe, 1981).

Much attention has been paid to the pathogenetic role of various antibodies and immune complexes in the development of RV (Fauci, Haynes & Katz, 1978). Subendothelial deposits of immunoglobulins are always present in biopsy specimens of affected skin of RV patients (Pernis, Ballabio & Chiappine, 1963). In unaffected skin of RA patients, a correlation was found between subendothelial immunoglobulin deposition and the presence of a perivascular cell infiltrate (Westedt *et al.*, 1984). Patients with RV have higher titres of circulating immune complexes than do patients with uncomplicated RA, and a positive correlation has been reported between the titre of circulating immune complexes and the occurrence of extra-articular manifestations of RA (Westedt *et al.*, 1985). However, the mechanisms by which circulating immunoglobulins lead to vasculitis, and the exact target of immune vascular damage are not known. Because it forms the interface between circulating blood and the vessel wall, the endothelial cells could well be the initial site of immune vascular damage. In the present study, we investigated the ability of antibodies in the serum of RV patients to bind to human umbilical vein endothelial cells.

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MATERIALS AND METHODS

Patients

Serum samples were collected from 28 patients with RV; all these patients had classical RA, and had leucocytoclastic vasculitis proven by skin biopsy. Serum samples were also obtained from 24 patients with RA without signs of vasculitis, and from 34 healthy donors. We also studied the serum of 10 patients with systemic lupus erythematosus (SLE). RA, RV and SLE patients all fulfilled the criteria of the American Rheumatism Association. The RA and RV patient groups were matched for age, sex ratio and disease activity. Patients with RV had significantly higher levels of circulating immune complexes ($P < 0{\cdot}0001$). Serum samples were stored at $-20°C$ until use and thawed two times at most.

Cells

Endothelial cells were isolated and cultured as previously described (Breedveld *et al.*, 1988), and serially passaged (passage level 3–8) as described by Thornton, Mueller & Levine (1983). Endothelial cells were positive for factor VIII antigen. Umbili-

cal cord fibroblasts and lung fibroblasts were isolated according to standard methods. For the adsorption studies, peripheral blood erythrocytes (Diepenhorst & Engelfriet, 1975), lymphocytes, granulocytes, and monocytes were isolated as described elsewhere; the purity of the cell suspensions was at least 95% (Claas *et al.*, 1983). Thrombocytes were isolated as described previously (Brand *et al.*, 1978).

Sera testing

Rheumatoid factors were determined by the Rose–Waaler test according to established methods (Rose *et al.*, 1949) and anti-nuclear antibodies (ANA) were determined by indirect immunofluorescence (Meyer *et al.*, 1982). For the assessment of immune complexes in the sera, use was made of the C1q binding assay (Kauffmann, van Es & Daha, 1979). Immune complexes were precipitated by adding polyethylene glycol (PEG 6000, BDH Chemicals, Poole, UK) to sera, to obtain a final concentration of 3·4%, followed by incubation (1 h at 0°C) and centrifugation. Information about the size of the molecules with anti-endothelial antibodies (AEA) activity was obtained by fractionating whole serum of healthy donors and RA and RV patients by gel filtration on sephacryl S-300 (Pharmacia, Woerden, The Netherlands) columns as described previously (Hiemstra *et al.*, 1988) and testing of these fractions for AEA activity. Fractions were collected and total protein concentration was determined in each fraction by the folin method (Lowry *et al.*, 1951).

IgG was purified from the serum of RV and RA patients by anion exchange chromatography making use of diethylaminoethyl (DEAE)-sephacel (Gorter *et al.*, 1987). $F(ab')_2$ fragments of patient IgG were prepared by pepsin digestion (Breedveld *et al.*, 1985).

Adsorption studies

Sera of several selected RV, RA, and SLE patients were adsorbed with cells of various types. Undiluted serum (200 μl) was incubated with an equal volume of packed thrombocytes (Rodey, Sturm & Aster, 1976), granulocytes, lymphocytes, or erythrocytes. For removal of AEA activity from purified preparations of IgG, use was made of either endothelial cells (from four donors) or fibroblasts. IgG from each source was incubated with an equal volume of packed cells for 1 h at 37°C and then for 18 h at 4°C before being tested for AEA activity on endothelial cells and fibroblasts.

Anti-nuclear antibodies (ANA) were absorbed from serum of RV and SLE patients with the use of permeabilized chicken erythrocytes (Searles, Messner & Bankhurst, 1979).

Antibodies for the detection of AEA

Rabbit IgG reactive with human-$F(ab')_2$ was prepared in our laboratory and used to detect endothelial cell-bound immunoglobulins. For detection of the IgG–AEA we used mouse monoclonal anti-human IgG (HB 43, American Tissue Type Culture Collection, Rockville, MD).

Determination of antibodies directed against endothelial cells

For each experiment in both methods described below, 10^4 endothelial cells/well were plated in 96-well, flat-bottomed microtitre plates (Costar, Cambridge, MA) and used after 2–3 days when they had reached confluence.

Radioimmunoassay (RIA). Endothelial cell monolayers were subsequently incubated with 100 μl serum diluted 1:25 in HBSS/0·5% bovine serum albumin (BSA) for 1 h at 37°C in 5% CO_2 and with biotinylated (Zymed, San Francisco, CA) mouse monoclonal anti-human IgG (HB 43). Since linear dose responses were found with positive sera, and because 1:25 dilution of the sera gave values appropriate for RIA and ELISA, the 1:25 dilution was used for the screening of all sera. Thereafter, the monolayer was incubated with 100 μl ^{125}I-streptavidin (Zymed) for 1 h at 37°C in 5% CO_2. Labelling of streptavidin with ^{125}I has been described elsewhere (Thorell & Larsson, 1974). Cells were removed from the wells with 1 M NaoH and the percentage bound radioactivity was determined in a gamma spectrophotometer (LKB, Wallac 1277 Gammamaster, Woerden, The Netherlands). Each experiment was performed in triplicate; s.d. were $<10\%$. All assays included at least one positive and one negative control sample on each plate.

For comparison of the results of the assays performed on different days and with endothelial cell monolayers from different donors, an antibody binding index was calculated as:

$$\frac{\text{ct/min X} - \text{ct/min HBSS–0·5\% BSA}}{\text{ct/min HD} - \text{ct/min HBSS–0·5\% BSA}}$$

where X represents patient serum and HD represents healthy donor serum. Sera were defined as positive when the binding activity exceeded the mean + 3 s.d. of the healthy donor values.

ELISA. Incubation of endothelial cell monolayers with serum and biotinylated HB 43 were performed as under RIA. Binding was determined using peroxidase-conjugated streptavidin (Zymed) and ortho-phenylenediamine as a substrate for the peroxidase. Each experiment was performed in triplicate; s.d. were $<10\%$. A ratio was calculated as:

$$\frac{\text{ODX} - \text{ODHBSS–0·5\% BSA}}{\text{ODHD} - \text{ODHBSS–0·5\% BSA}}$$

where X represents OD of patient serum, and HD, of healthy donor. A serum was considered positive when the percentage of binding exceeded the mean + 3 s.d. healthy donor values.

Stastical analysis

The Mann–Whitney *U*-test was used to compare the differences in AEA activity between several patient groups (RV, RA and SLE) and the healthy donors.

RESULTS

Binding of immunoglobulin to cultured endothelial cell monolayers

With the RIA, AEA activity was detected in 21 (75%) of the 28 patients with RV, in three (13%) of the 24 with RA, and in seven (70%) of the 10 SLE patients. Samples of healthy donor serum showed no detectable AEA activity. The percentage of patients with AEA activity was significantly higher for RV and SLE than for RA and control ($P < 0·0001$). We also developed an ELISA. With this method, AEA activity was found in 19 (68%) of the 28 RV patients, in four (16%) of the 24 RA patients; and in seven (70%) of the 10 patients with SLE. No AEA activity was found in the sera of healthy donors (Fig. 1). Compared with binding in the healthy donors, that in RV patients' sera was significantly increased (mean level four-fold over healthy donors in RIA and three-fold over healthy donors in ELISA). To assess the

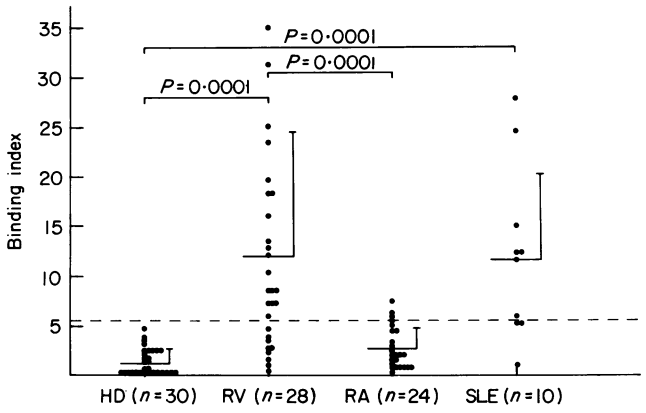

Fig. 1. IgG-anti endothelial cell antibody (IgG–AEA) activity in serum of healthy donors (HD), and patients with rheumatoid vasculitis (RV), rheumatoid arthritis (RA), or systemic lupus erythematosus (SLE) assessed with an ELISA. The IgG–AEA activity was measured and expressed as the mean values of triplicate determinations and recalculated to binding indices. All s.d. are < 10%. The horizontal dashed line represents the level of the mean value of the binding index for the HDs plus three times the s.d.

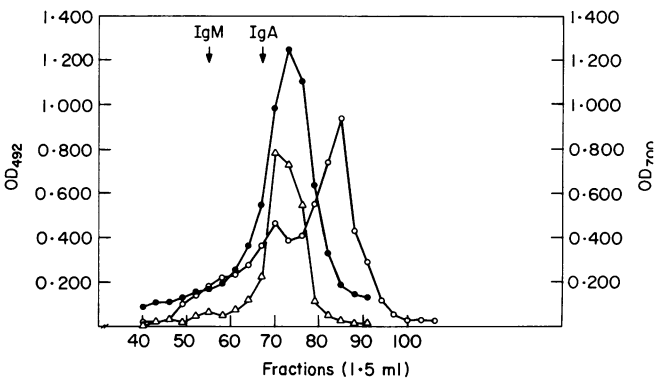

Fig. 2. The gel filtration profile for serum IgG-anti endothelial cell antibody activity (IgG–AEA). The serum of a patient with rheumatoid vasculitis (RV) was applied to a sephacryl S-300 column, and the IgG, IgA and IgM concentration in each fraction was determined by ELISA. Total protein concentration was measured by the folin method. The IgG–AEA activity was determined by ELISA. The fractions containing peak amounts of IgA and IgM are indicated with arrows. △, AEA activity; ○, protein profile; and ●, IgG profile.

reproducibility of the assays employed, we tested two AEA-positive and two AEA-negative sera with the RIA on seven endothelial cell monolayers and with the ELISA on five endothelial cell monolayers originating from different donors. With all monolayers tested, sera with AEA activity gave positive results in both ELISA and RIA and the AEA-negative sera always gave negative results.

To find out whether AEA activity represents binding of monomers of IgG or IgG in immune complexes, the RIA was performed in the supernatants of sera of 20 RV patients and five SLE patients after precipitation with PEG. The corresponding non-PEG-treated sera were tested at the same time. For the serum of five RV patients, the C1q binding assay values dropped from 331±194 μgeq/ml before to 2±5 μgeq/ml after PEG precipitation. However, no significant differences were found between the AEA binding activity of the sera before and after precipitation of immune complexes with PEG.

Further proof of the presence of IgG–AEA was obtained from the results of gel filtration of sera of two RV patients and one healthy donor on a sephacryl S-300 column. Figure 2 shows a representative example of the results. AEA activity, total IgG, IgA and IgM, and protein concentration were determined in all fractions. The highest AEA activity was found in the fractions with the highest concentration of IgG. To investigate the binding characteristics of AEA further, we purified IgG from the sera of three RV and three RA patients and one healthy donor by DEAE anion exchange chromatography. The ELISA studies showed AEA activity in the purified IgG of all RV patients and one RA patient. When endothelial cells were incubated with $F(ab')_2$ fragments from the IgG of these patients, AEA activity remained positive. After pepsin digestion AEA activity amounted to 60–87% of the AEA activity found with undigested IgG. No AEA activity was found with $F(ab')_2$ fragments of IgG from sera lacking AEA activity.

Specificity of AEA

The cell specificity of AEA in serum was investigated in AEA-positive samples of six RV patients and four SLE patients, with the use of endothelial cells and fibroblasts from the same umbilical cord. AEA-negative samples of five RA patients and four healthy donors served as controls. The AEA-positive samples showed binding to fibroblasts, whereas the AEA-negative samples were also negative for fibroblasts. Similar results were obtained when the samples were tested with lung fibroblasts. When isolated IgG fractions of RV patients were adsorbed on endothelial cells or fibroblasts, the binding of IgG to both endothelial cells and fibroblasts decline in parallel (Table 1). Total IgG levels determined in the sera before and after adsorption experiments did not differ significantly, which rules out dilution effects.

Table 1. Adsorption of IgG–AEA activity to endothelial cells and fibroblasts

	Tested on	
Adsorption with	Endothelial cells	Fibroblasts
No cells	100	100
Endothelial cells	50±5	39±4
Fibroblasts	51±2	44±2

IgG fractions of serum from three patients with rheumatoid vasculitis were incubated with endothelial cells, fibroblasts, or with no cells. The supernatants were then (cross-) tested for IgG binding activity on endothelial cell and fibroblasts monolayers.

Results are expressed as mean percentages ±1 s.d. of the antibody activity that remained in the IgG fractions, as calculated from the OD values (ELISA).

AEA, anti-endothelial antibodies.

Table 2. IgG-AEA activity of sera of one SLE patient and two RV patients before and after adsorption of ANA with permeabilized chicken erythrocytes

Patient no.	Diagnosis	Binding index	
		Before adsorption	After adsorption
1	SLE	7·2	12·7
2	RV	10·1	10·8
3	RV	8·5	10·9

Sera were tested for IgG binding activity on endothelial cell monolayers (ELISA). Values are expressed as a binding index. A value of more than 5·5 was considered positive for IgG-AEA activity. ANA in the sera were positive before and negative adsorption with permeabilized chicken erythrocytes.

AEA, anti-endothelial antibody; SLE, systemic lupus erythematosus; RV, rheumatoid vasculitis; and ANA, antinuclear antibodies.

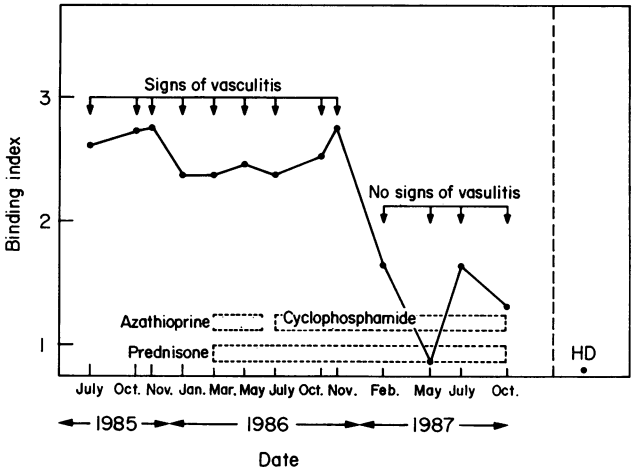

Fig. 3. Association of the clinical signs of vasculitis with the presence of serum IgG-anti endothelial cell antibody activity in a 60-year-old man with rheumatoid arthritis who developed signs of vasculitis. Treatment consisted of prednisone, azathioprine, and cyclophosphamide. There is an association between the titre of AEA and the clinical signs of vasculitis. All serum samples were tested by RIA on one day and with endothelial cells derived from one healthy donor (HD).

Serum samples with AEA activity from two patients with RV were also adsorbed with haematopoietic cells. The percentage of AEA-adsorption ranged between 0 and 8% with erythrocytes, 0 and 16% with granulocytes, 8 and 11% with lymphocytes, and 27 and 36% with thrombocytes.

For the studies to find out whether AEA activity was related to serum ANA activity, three serum samples with AEA activity from two RV patients one SLE patient were absorbed with permeabilized chicken erythrocytes to remove ANA. No loss of AEA activity was found after adsorption of ANA (Table 2).

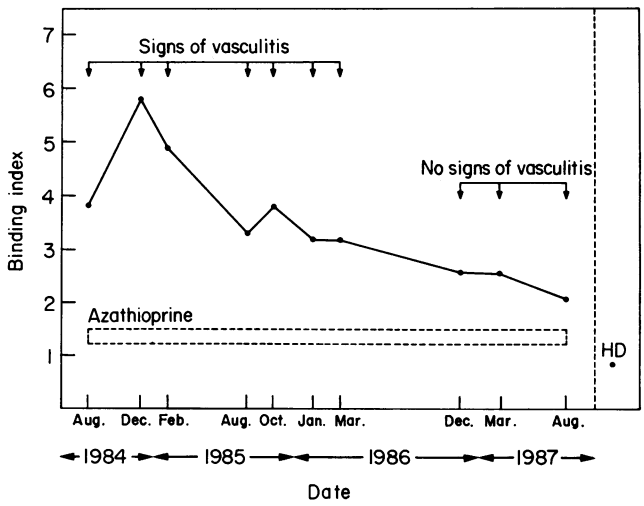

Fig. 4. Association of the clinical signs of vasculitis with the presence of serum IgG-anti-endothelial antibody activity in a 69-year-old woman with rheumatoid arthritis who developed signs of vasculitis. Treatment consisted of azathioprine. Over a period of 3 years the titre of AEA declined and the signs of vasculitis disappeared. Serum sample tested as in Fig. 3.

Regression of vasculitis episodes tended to correlate with decreasing titres of AEA

Sera from four patients with RV followed longitudinally were investigated with respect to AEA activity in relation to disease activity. The results of two representative examples are shown in Figs 3 and 4. In all patients the AEA titre declined in parallel with disappearance of the clinical signs of vasculitis.

DISCUSSION

Our findings show that IgG-AEA occur in sera of patients with RV. These antibodies could be detected reproducibly with RIA and ELISA techniques. A small proportion (13–16%) of the sera of RA patients without clinical signs of vasculitis showed IgG-AEA activity too. None of the healthy donors sera displayed AEA activity. IgG-AEA are monomeric, as shown by the results of gel filtration. Furthermore, IgG isolated by anion exchange chromatography contained AEA activity and this activity was also found in $F(ab')_2$ preparations of these isolated IgG fractions.

The AEA-binding activity detected in our assays was not specific for endothelial cells. All samples positive on endothelial cells and tested on fibroblasts also showed antibody activity on these cells. This suggests that AEA represents a group of autoantibodies directed to a variety of different antigens on different cells. An alternative explanation may be that endothelial cells and fibroblasts share certain antigens. The latter hypothesis is supported by the finding that cross-adsorption with endothelial cells or fibroblasts leads to a decrease of AEA on both types of cells. Further studies on the characterization of the antigens involved are in progress.

The AEA activity is not directed to either ABO antigens or HLA-associated antigens. Adsorption experiments with erythrocytes, lymphocytes, and granulocytes obtained from several healthy donors showed no significant loss of AEA activity.

Adsorption of sera on thrombocytes, however, gave a loss of AEA activity amounting to as much as 36%. This might be due to the presence of similar antigens on thrombocytes and endothelial cells or to non-specific adsorption of AEA activity. Since ANA may also react with intact cells (Horneland, Rekvig & Jørgensen, 1983), the positive results in the AEA assay could also be explained by the presence of ANA. However, adsorption of ANA activity of sera of two RV patients did not influence IgG binding to endothelial cells. Furthermore, several RA patients without AEA activity had high titres of ANA.

Antibodies binding to endothelial cells has been described by several investigators in various rheumatic diseases. Cines *et al.* (1984) reported IgG-AEA in SLE patients. Later studies showed AEA in patients with progressive systemic sclerosis (Hashemi, Smith & Izaguirre, 1987; Rosenbaum *et al.*, 1988), SLE (Shingu & Hurd, 1981; Hashemi *et al.*, 1987), Kawasaki syndrome (Leung *et al.*, 1986), and in children with acute haemolytic uraemic syndrome (IgG and IgM) (Leung *et al.*, 1988). Rosenbaum *et al.* (1988) reported IgG-AEA in 11 of 39 RA patients. The results of the present study confirm these findings and serve to contribute to the suggestion that AEA activity is present much more frequently in RV patients than in patients with uncomplicated RA.

Injury to endothelial cells mediated by AEA could be a primary event in the pathogenesis of vasculitis. In patients with Kawasaki syndrome, Leung *et al.* (1986) described cytotoxic AEA of the IgM class, and in these patients the serum AEA levels correlated positively with clinical signs of vasculitis. This cytotoxic effect was only seen when endothelial cell monolayers were pretreated with interferon-gamma. At present we are investigating the effects of AEA on the endothelial cells and on the interaction between endothelial cells and inflammatory cells. We have found no evidence yet for complement-mediated cytotoxicity on interferon-gamma-pretreated endothelial cells.

The results of the present study show that IgG-AEA are present four times more frequently in patients with RA complicated by vasculitis than in patients with only RA. In four patients there was a trend for lower titres of antibodies to correlate with regression of vasculitis episodes. These findings suggest a role of AEA in the pathogenesis of RV. The assays used to detect AEA are relatively easy to perform and the results are reproducible. The detection of AEA therefore may also be a valuable tool for the diagnosis of vasculitis in patients with RA.

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