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Anti-endothelial cell antibodies in sera of patients with autoimmune diseases: comparison between ELISA and FACS analysis

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SUMMARY

In some patients suffering from rheumatoid arthritis (RA), vasculitis is a clear clinical manifestation, mentioned as rheumatoid vasculitis (RV). Autoantibodies directed against endothelial cells (AEA) have been implicated in the pathogenesis of this disorder, and it has been suggested in a number of studies that testing for AEA should be included in diagnosing RV. To test this hypothesis, we have evaluated the presence of AEA in sera of patients suffering from various autoimmune diseases, employing an ELISA with fixed cultured endothelial cells (EC). In all the groups of patients ELISA-positive sera were present. A significant difference in percentage of positivity was found between the RA and RV group ($P < 0.05$). In addition, our results indicated that not only antibodies directed against antigens on the EC membrane were detected, but also antibodies directed against intracellular components like DNA, histones and cytoskeletal components. Therefore, we also tested all these patient sera on unfixed intact EC using indirect immunofluorescence followed by FACS analysis. Whereas in the total patient population 34 out of 65 patients were AEA-positive as determined in the ELISA, only seven patients were weakly positive when examined by flow cytometry. We conclude that: (i) an ELISA on fixed EC does not specifically detect AEA. A positive test result is, however, to some extent correlated with the occurrence of vasculitis, and may therefore be helpful in diagnosing this disease; (ii) FACS analysis is a more suitable method than ELISA to measure the presence of membrane-specific AEA in patient sera; (iii) specific IgG-AEA are less common in patients suffering from autoimmune disorders than was assumed previously.

Keywords endothelium vasculitis ELISA FACS rheumatoid arthritis

INTRODUCTION

Antibodies directed against endothelial cells (AEA) occur during various pathological conditions. During allograft rejection, an antibody response against endothelial cells is mounted that may play a role in the effector phase of the rejection process [1]. In several autoimmune diseases autoantibodies directed against endothelial cells (EC) have been described. These diseases include systemic lupus erythematosus (SLE), scleroderma (Ssc), Wegener's granulomatosis, mixed connective tissue disease (MCTD), haemolytic uraemic syndrome, Sjögren's syndrome, rheumatoid arthritis (RA) and rheumatoid vasculitis (RV) [2–6]. Although the vascular damage that is frequently observed in SLE and other patients is thought to be mediated mainly by immune complexes [7], autoantibodies directed against EC have also been implicated in this process [6,8–12]. The observation that anti-endothelial cell antibodies (AEA) are found more often in RV patients than in RA supports an involvement of AEA [5,13]. The presence of AEA in sera of these groups of autoimmune patients may thus be a marker for vasculitis or vascular damage. Measurement of AEA may therefore facilitate the diagnosis of this disorder, which could until now only be made unambiguously by histopathological examination of a skin biopsy. In the present study we tested this hypothesis by measuring AEA levels in SLE, RA, RV, MCTD and Ssc patients, and in a group of healthy controls. We determined IgG AEA levels with an ELISA employing fixed cultured human umbilical vein endothelial cells (HUVEC), and measured in parallel experiments the amount of AEA bound to unfixed HUVEC via indirect immunofluorescence and flow cytometric analysis. We compare the results of both methods of AEA measurement, discuss the

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Table 1. Characteristics of patients examined

	Patient groups						
	RA	RV	SLE (1)*	SLE (2)*	MCTD	Ssc	Controls
n	19	11	12	3	12	8	15
Mean age in years (range)	66 (43–84)	57 (36–68)	35 (20–49)	38 (29–41)	41 (27–70)	52 (41–71)	27 (21–44)
Male/female	5/14	5/6	3/9	3/0	2/10	2/6	7/8
RF positive (*n*)	16	9	2	0	0	3	0
Anti-dsDNA positive (*n*)	0	0	12	0	1	0	0
C1q-binding positive† (*n*)	10	6	6	0	5	3	0
Anti-RNP positive (*n*)	ND	ND	ND	ND	12	ND	ND

*SLE (1), Anti-dsDNA antibodies present; SLE (2), anti-dsDNA antibodies absent.
†C1q-binding positive: > 5 s.d.
ND, Not determined.

differences, and comment on the determination of AEA as a diagnostic tool in defining the pathological status of autoimmune patients suspected of suffering from vasculitis.

PATIENTS AND METHODS

Patients

Characteristics of the patient groups tested in this study are summarized in Table 1. Serum samples were obtained from 19 patients with RA according to the 1987 revised criteria of the American Rheumatism Association (ARA) for RA [14], clinically not complicated by vasculitis, and 11 patients with RA complicated by vasculitis. Histopathological confirmation of vasculitis was obtained by skin biopsy analysis, performed in all patients. Serum samples were also obtained from 15 patients with SLE diagnosed using the ARA criteria [15]. The serum of 12 of these patients contained anti-double stranded (ds) DNA (SLE (1)), as determined by an immunofluorescence test in *Crithidia lucillae* and quantified by Farr assay [16], and three without anti-dsDNA (SLE(2)). C1q binding assay was performed as described by Zubler *et al.* [17]. Anti-RNP antibodies were detected by an immunoblotting assay as described previously [18]. Finally, serum samples were collected from 12 patients with MCTD according to the criteria of Sharp *et al.* [19], eight patients with Ssc [20] and 15 healthy controls.

Cells

HUVEC were isolated by collagenase treatment essentially as described previously [21]. Cells were subcultured at 37°C and 5% CO_2 in Eagle's modified essential medium (EMEM; Flow Labs, Rickmansworth, UK), supplemented with 15% heat-inactivated human serum, 15% fetal calf serum (FCS; Gibco BRL, Paisley, UK), 150 μg/ml endothelial cell growth factor (isolated from calf brain as described before [22]), 2 mM glutamine, 5 U/ml heparin (Organon Teknika, Boxtel, The Netherlands), and antibiotics in fibronectin-coated tissue culture flasks (Costar, Cambridge, MA). Cells were detached from culture flasks using a solution of 0·125% trypsin (Difco, Detroit, MI) in 0·2% EDTA/PBS. In some experiments, EC were treated for 3 days with 200 U/ml interferon-gamma (IFN-γ; a kind gift from Boehringer Ingelheim, Ingelheim, Germany), to enhance the expression of MHC class I molecules.

Antibodies

Mouse MoAb W6/32 (anti-HLA class I) and HB43 (anti-human IgG) were obtained from ATCC (Rockville, MD), Anti-TCR MoAb WT31, anti-DNA MoAb 42 and anti-histone MoAb KM2.4 were kind gifts from Dr W. Tax, Dr R. Termaat and Dr K. Kramer (Department of Nephrology, University Hospital, Nijmegen), respectively. Anti-RNP MoAbs SW5 and 436 were a kind gift from Dr W. van Venrooy (Department of Biochemistry, Faculty of Science, University of Nijmegen). MoAbs RV202, RD301 and RCK102, RCK105 and RCK106 (anti-vimentin, -desmin and -cytokeratin, respectively) were developed in our laboratory and described previously [23,24]. Anti-endoglin MoAb PN-E2 was also developed in our laboratory and described recently [25]. Polyclonal human allo-antibodies directed against human MHC class I (HLA-Bw4/B46) (obtained from multiparous women) were a kind gift from Dr H. Bruning (Leiden, The Netherlands).

ELISA

An ELISA to detect AEA in human sera was performed as described previously [26], with some modifications. Briefly, HUVEC were seeded in gelatine-coated 96-well microtitre plates (Costar) at 2×10^4 cells/well, and allowed to grow to confluence for 3 days. Cells were fixed with 0·025% glutaraldehyde in PBS for 10 min at room temperature, and incubated with blocking buffer (1% gelatine/1% bovine serum albumin (BSA) in PBS) for 30 min at 37°C to prevent non-specific binding of antibody. After washing with PBS the wells were successively exposed for 60 min at 37°C to patient serum, HB43, and polyclonal alkaline phosphatase-labelled rabbit anti-mouse immunoglobulin heavy and light chain antibody (Dako, Glostrup, Denmark), both diluted appropriately in PBS/1% BSA. Substrate for the enzyme (*p*-nitrophenyl phosphate, Sigma, St Louis, MO) was then added in 1 M diethanolamine/0·5 mM $MgCl_2$. Colour development was measured after 60 min by reading the OD at 405 nm in a Titertek ELISA plate reader (Flow). The degree of specific MoAb binding to EC was calculated by subtracting the OD values of gelatine-coated microtitre wells without EC incubated with patient serum, HB43 and RαM-Ig-PO from all test values. Sera were tested in a concentration range from $1:10^1$ to $1:10^4$.

All sera were tested at least in triplicate. ELISA measurements were quantified by determining the area enclosed by the extinction curve, the abscissa, and vertical lines at serum dilution $1:10^1$ and $1:10^4$ (area under the curve, AUC) in arbitrary units. The mean AUC of the 15 healthy control sera was 35 ± 23 units (range 0–67) The mean + 3 s.d. was taken as the threshold for positivity (104 units).

Indirect immunofluorescence

HUVEC were detached from culture flasks non-enzymatically, washed and incubated in suspension with undiluted patient serum at 5×10^5 cells per incubation at 4°C for 2 h. After removing unbound antibody by washing three times with PBS/1% BSA/0·1% NaN_3, cells were stained with FITC-conjugated sheep anti-human IgG. Cells were subsequently fixed in 1% paraformaldehyde, and analysed using a flow cytometer (Coulter).

Statistical analysis

Test results from the AEA-ELISA were statistically evaluated using the Wilcoxon rank sum test, and results from the FACS analysis with Fisher's exact test.

RESULTS

AEA in patient sera measured by ELISA

We measured the amount of AEA in human sera in an ELISA on fixed cultured EC. The intra-assay variability of this ELISA was studied with one serum from an RA patient (tested eight times in the same experiment) and a pool of sera from 52 healthy controls (tested six times in the same experiment). The results of the ELISA were quantified by determining the AUC in arbitrary units. The AUC of the patient serum was 506 ± 150 units (range 259–735), and the AUC of the control pool was 40 ± 34 units (range 0–97). The interassay variability was comparable to the intra-assay variability (data not shown).

Using this assay we analysed the AEA reactivity of 65 sera obtained from patients with various autoimmune diseases, and of 15 sera obtained from healthy controls. Remarkable differences were already observed in the binding patterns to control gelatine-coated microtitre wells without EC. Sera reacting strongly with the gelatine coating were found both in the healthy control and in the patient groups. The significance of this reactivity was unclear. No correlation was found with the strength of the specific anti-EC signal (data not shown). Optical density values obtained from gelatine-coated wells without EC were subtracted from values obtained with EC to obtain specific anti-EC reactivity.

In Fig. 1 the resulting AUC values of the tested sera are shown (the difference between filled, semi-filled and open circles will be discussed below). The 15 separately tested healthy control sera yielded an AUC value of 35 ± 23 units (0–67). The mean + 3 s.d. (104 units) was taken as a threshold for positivity.

The different patient groups displayed different patterns of AEA positivity. In the RV group, statistically significantly more patients were found AEA positive (seven out of 11 patients, 64%) than in the RA group without vasculitis (two out of 19 patients, 11%), as determined with the Wilcoxon rank sum test ($P < 0.05$). Of 12 SLE patients with anti-DNA antibodies, 11 were positive in the anti-EC ELISA (91%), and two out of three SLE patients without anti-DNA antibodies were positive. No correlation between the amount of anti-dsDNA antibodies and the AUC level was observed in the anti-dsDNA-positive group (data not shown). Finally, in the Ssc and the MCTD group five out of eight (63%) and seven out of 12 (58%) patients, respectively, were positive in the anti-EC assay.

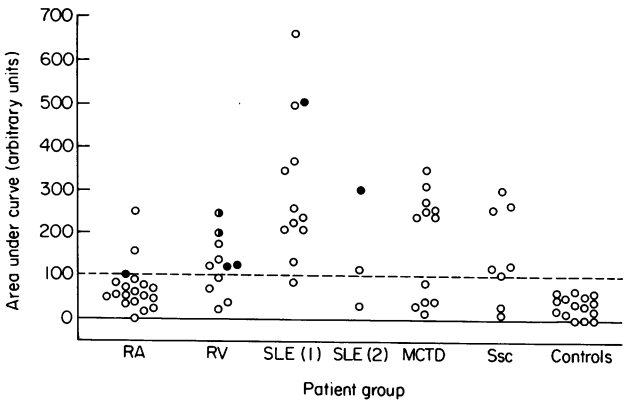

Fig. 1. Anti-endothelial cell antibody (AEA) reactivity in 80 human sera as determined by ELISA on fixed endothelial cells. AEA reactivity is expressed as area under the curve (AUC) of the ELISA graph in arbitrary units as described in Patients and Methods. The mean AUC of 15 healthy control sera +3 s.d. (104 units) was taken as a threshold for positivity in the assay (indicated by dotted line). The filling of the circles represents AEA positivity of an individual sample as determined by indirect immunofluorescence on unfixed endothelial cells followed by flow cytometric analysis. ○, Negative; ●, positive; ◑, subpopulation of endothelial cells (10%) positive. SLE (1), Anti-dsDNA antibodies present in patient serum; SLE (2), no anti-dsDNA antibodies present in patient serum. RA, Rheumatoid arthritis; RV, rheumatoid vasculitis; MCTD, mixed connective tissue disease; Ssc, scleroderma.

Detection of intracellular components in the ELISA with fixed EC

The high incidence of AEA-positive sera in the SLE group raised the question whether anti-DNA antibodies might have caused positive signals in the AEA-ELISA. To determine the specificity of the AEA-ELISA, we tested MoAbs directed at a number of endothelial membrane molecules on the one hand, and endothelial intracellular components on the other. Anti-HLA class I MoAb W6/32 (data not shown) and anti-endoglin (a transforming growth factor-beta (TGF-β) binding protein with a high expression level on cultured EC) MoAb PN-E2 showed a high binding level to fixed EC, whereas control MoAb WT31 (anti-TCR) was negative (Fig. 2). However, MoAbs directed against two nuclear antigens (histone, Fig. 2a, and double-stranded DNA, Fig. 2b) were clearly positive as well. Anti-RNP MoAb SW5 (anti-La, Fig. 2c) and 436 (anti-B″, data not shown) on the other hand, were negative. Of antibodies directed against cytoskeletal components cytokeratin, desmin and vimentin, only anti-vimentin MoAb RV202 showed a weak but distinct reactivity in the EC-ELISA, corresponding with the cytoskeletal phenotype described for endothelial cells (Fig. 2d).

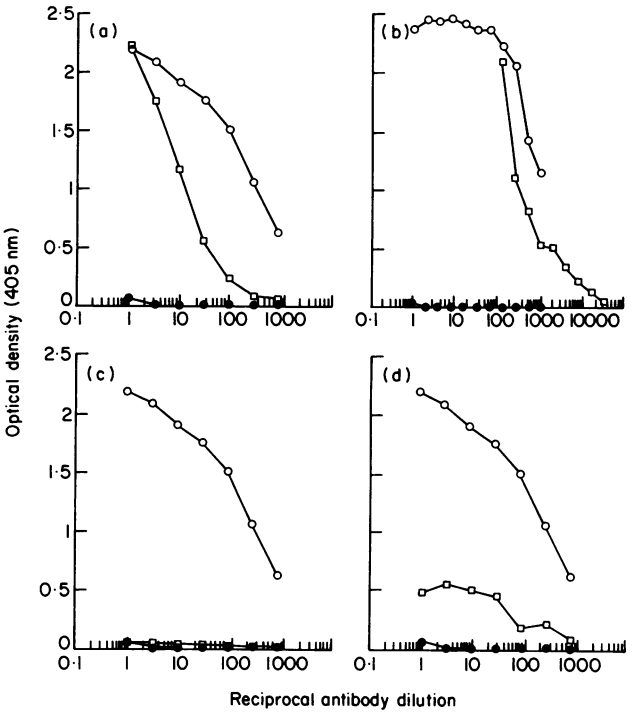

Fig. 2. Reaction of MoAbs to fixed endothelial cells in ELISA. Positive control MoAb: PN-E2 (anti-endoglin; ○); negative control MoAb: WT31 (anti-TCR; ●); MoAb directed against intracellular component (□). (a) MoAb KM2.4 (anti-histone). (b) MoAb 42 (anti-dsDNA). (c) MoAb SW5 (anti-La). (d) MoAb RV202 (anti-vimentin).

Detection of AEA on unfixed EC with immunofluorescence and FACS analysis

Our data led us to question whether antibodies in human sera binding to fixed EC in our ELISA were directed at intracellular molecules of the endothelium rather than at membrane-bound molecules. Attempts to perform an ELISA with unfixed EC were unsuccessful (data not shown). As an alternative, we investigated the binding of human antibodies to unfixed endothelial cells by indirect immunofluorescence followed by flow cytometry analysis. As a control, we analysed the binding of antibodies directed against HLA class I antigens (Bw4/B46) present in a human alloantiserum. Both untreated and IFN-γ-treated endothelial cells were tested, and the results were compared with conventional immunofluorescence analysis employing mouse MoAb directed against a monomorphic determinant of HLA class I molecules. As demonstrated in Fig. 3, the human and the mouse antibodies stained the endothelial cells in a comparable fashion. In both instances, the up-regulation of the HLA class I molecule expression by the IFN-γ treatment could be clearly observed. Next, we examined the binding of the MoAb we also tested in the ELISA to unfixed EC. Antibodies directed against cytoskeletal components or anti-RNP MoAb were negative (data not shown). Anti-endoglin MoAb PN-E2 was highly positive, whereas anti-histone MoAb KM2.4 showed a weak staining. Anti-dsDNA MoAb 42 was negative (data not shown). Finally, we tested the patient sera and the healthy control sera on unfixed

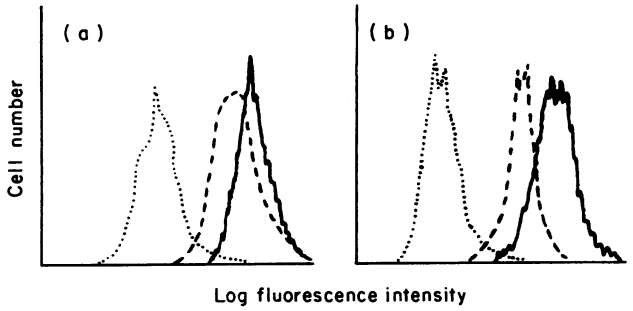

Fig. 3. Binding of anti-MHC class I antibodies to unfixed endothelial cells (EC) determined by indirect immunofluorescence followed by flow cytometric analysis. (a) EC incubated with human alloantibodies, followed by FITC-labelled anti-human IgG. (b) EC incubated with mouse MoAb W6/32, followed by FITC-labelled anti-mouse IgG serum. . . . . , Unstimulated EC incubated with FITC-labelled anti-mouse IgG or anti-human IgG only (background controls); – – – –, unstimulated EC; ————, EC stimulated by treatment for 3 days with 200 U/ml IFN-γ to enhance MHC class I expression.

cells. Scatterplots of the EC samples showed that the proportion of dead EC in samples treated with control sera was identical to the proportion of dead cells in samples treated with patient sera (10% or less). This indicated that EC viability was not influenced by cytotoxicity of AEA.

All healthy control sera were negative. Whereas from 65 patient sera 34 sera were positive in the AEA-ELISA, only seven sera showed a weak reaction with the unfixed EC (filled circles in Fig. 1). The FACS profiles of these seven patients are shown in Fig. 4. Of two RA sera positive in the ELISA, none was positive on unfixed cells. One RA serum negative in the ELISA (103 AUC units), however, was weakly positive on unfixed cells. Four out of seven ELISA-positive RV patients were also reactive with unfixed cells. Two of these sera (semi-filled circles in Fig. 1), however, showed a strong reaction with only a subpopulation (<10% of the cells) of the EC. No statistical significance was observed between the number of AEA-positive patients in the RA and RV group, as determined by Fisher's exact test. Only one of the 11 sera of SLE patients with anti-dsDNA that were positive in the ELISA also reacted with unfixed cells. No sera from the Ssc or MCTD group reacted with unfixed cells.

DISCUSSION

The current study was undertaken to investigate the presence of IgG in sera of patients with autoimmune phenomena, with a previously described ELISA employing fixed cultured EC. We introduced some technical modifications in the ELISA procedure, and in the processing of the test results. First, we tried to decrease the level of intra- and interassay variability by including the complete ELISA curve in the quantification of the AEA signal. This was done by measuring the AUC, rather than reading the optical density obtained at one dilution of the serum. In our hands this quantification method indeed decreased the intra- and interassay variability. Second, we found, somewhat surprisingly, a strong reaction of some sera, both in the patient and control group, with the gelatine coating used in the ELISA plate to facilitate the EC adhesion. In some

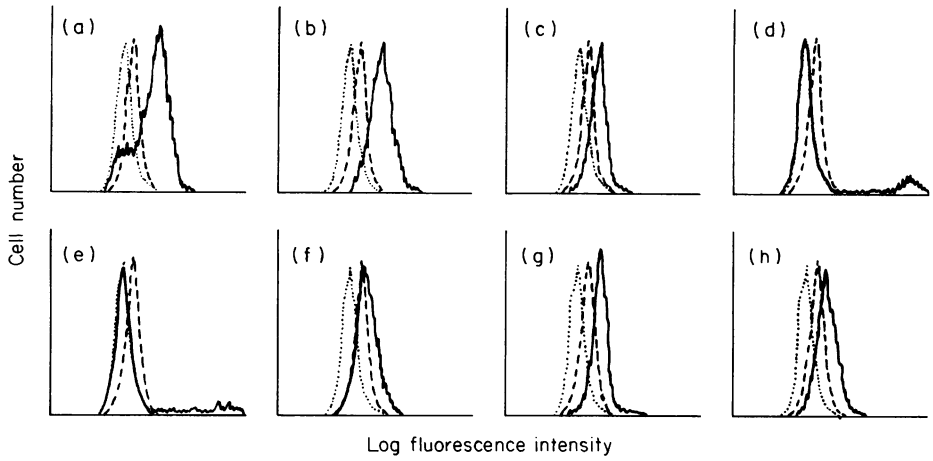

Fig. 4. Binding of anti-endothelial cell antibodies (AEA) present in patient sera to unfixed endothelial cells (EC) determined by indirect immunofluorescence followed by flow cytometric analysis. . . . . , Conjugate only (background control); – – – –, staining with serum pool of 52 healthy controls; ————, patient serum. (a) Allo-MHC class I antibodies. (b, c) Sera from rheumatoid arthritis (RA) patients (serum from patient in c: negative in AEA-ELISA). (d–f) Sera from rheumatoid vasculitis (RV) patients. (g, h) Sera from systemic lupus erythematosus (SLE) patients (g with, h without anti-dsDNA antibodies). (Sera correspond with filled circles in Fig. 1.)

instances, this signal even overcame the test values in the presence of EC, indicating that the antibodies were not EC-specific. Whether antibodies in these particular sera were sticking to the coating in a non-specific manner, or were reacting specifically with epitopes on the gelatine, is not clear. The latter is a serious possibility, since autoantibodies directed against collagen have recently been described in sera of SLE and vasculitis patients [27]. Whatever the cause of the reaction of some sera with gelatine may be, this control value clearly has to be subtracted on every occasion to exclude a false-positive outcome of the AEA determination in the ELISA.

We found IgG AEA-positive sera in all groups of autoimmune patients that we studied. The percentage of positive patients differed between the different disease categories. The highest incidence of positivity was found in the group of SLE patients with anti-dsDNA antibodies (91%). In RV, SSc and MCTD patients the percentage of AEA-positive patients was also considerable (58–63%). RA and RV patient groups showed significantly different percentages of AEA-positive patients (11% and 64%, respectively; $P < 0{\cdot}05$), although the number of patients in each group was relatively small. Our results suggest, however, that AEA determination with an ELISA on fixed EC as substrate indeed discriminates between RA and RV, confirming previously published data by van der Zee *et al.* [5].

When testing the specificity of our ELISA, however, we found that a number of MoAbs directed against intracellular components (double-stranded DNA, histones, and vimentin) were also reactive with fixed EC. Probably as a result of the fixation procedure, the interior of the EC became accessible to these antibodies. This implies that autoantibodies directed against intracellular components will also react in this type of ELISA, albeit in a non-specific manner. We decided that AEA-ELISA was not the method of choice to discriminate between these different types of autoantibodies. Assuming that only the antibodies directed at extracellular membrane-associated determinants will have relevance for *in vivo* antibody-mediated damage, we switched to indirect immunofluorescence using intact, non-fixed cells as a more reliable substrate to examine the binding of antibodies to membrane structures. When testing MoAbs in this system, anti-DNA and anti-vimentin were negative, whereas anti-histone MoAb KM2.4 showed a weak reaction. This latter reaction may be attributable to binding of MoAb to positively charged histone proteins derived from cellular debris that have bound to the negatively charged membrane of the endothelial cell [28–30]. Subsequently, we tested whether binding of human antibodies to unfixed EC could be detected. Human autoantibodies directed against allogeneic MHC class I molecules were easily detected, indicating that this method was useful for this purpose. The results differed dramatically from those obtained in the ELISA. Of the 34 patients that showed a positive reaction in the AEA-ELISA, only seven also showed detectable AEA on unfixed cells. Serum from one RA patient that was negative in the AEA-ELISA contained antibodies reactive with unfixed EC. It should be noted that the reaction of the AEA from the patient sera with the unfixed EC was relatively weak compared with the anti-MHC class I autoantibodies. Positivity in the immunofluorescence assay did not discriminate in a statistically significant way between RA and RV patients ($P > 0{\cdot}05$). Larger patient groups, however, will have to be investigated to verify this finding.

No correlation was found between the strength of the reaction of the AEA with the unfixed EC, and the degree of positivity in the AEA-ELISA. It therefore seems plausible that the reactivity of most of the patient sera in the AEA-ELISA is at least partly attributable to reaction of autoantibodies with intracellular components like DNA, histones or cytoskeletal proteins. Especially in the SLE sera, anti-DNA autoantibodies may react with fixed EC, thereby causing a false-positive outcome of the AEA determination. In a group of 12 SLE patients with anti-DNA antibodies, 11 patients were positive in the AEA-ELISA, whereas only one patient was positive in the immunofluorescence assay.

In conclusion, our results indicate that in the autoimmune diseases we investigated, IgG autoantibodies specific for mem-

brane molecules on EC may not be as common as has been assumed so far (values ranging from 28% to 67%; [5]). A prominent role for these antibodies as a possible mediator of the vascular damage observed in some of these disorders therefore seems less likely. A positive signal in the ELISA on fixed EC is not indicative for the presence of AEA, but is, nevertheless, to some extent correlated with the occurrence of vasculitis. Therefore, this assay may have value in diagnosing this disease. When one is determined to measure only AEA, however, the immunofluorescence method is more reliable than the AEA-ELISA.

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