

Comparison of cell-ELISA, flow cytometry and Western blotting for the detection of antiendothelial cell antibodies

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Abstract

Objective

There is still great uncertainty in the detection of antiendothelial cell antibodies (AECA). The aim of our study was to compare the results obtained using different methods.

Methods

Sera were obtained from 71 patients with a variety of vasculitides. Three assay methods were used: cell ELISA, flow cytometry (FACS) and Western blot (WB).

Results

In the ELISA 12/17 patients with systemic lupus erythematosus (SLE), 1/12 with Churg Strauss (CS) disease, 3/12 with micropolyarteritis (MPA) and 5/30 with Wegener's granulomatosis (WG) tested positive. Most of the sera that were positive on ELISA were not by FACS. Among the negative sera, 50% of WG, 40% of MPA, 20% of CS and 40% of SLE became positive on WB. There were some specific patterns of reactivity for a given disease, so that some bands could be assigned to a disease.

Conclusion

The discrepancies in the results may most probably be accounted for by differences between the antigenic preparations. Caution must thus be exercised when interpreting the results of any of these three tests.

Key words

Antiendothelial cell antibody, vasculitis.

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Introduction

Antiendothelial cell antibodies (AECA) have been described in a variety of autoimmune diseases (1), including systemic lupus erythematosus (SLE) and other disorders accompanied by systemic vasculitis, such as Wegener's granulomatosis (WG), micropolyarteritis (MPA), and Churg and Strauss disease (CS). In patients with WG, AECA are associated with active disease, while in those with SLE they are more frequent in the presence of renal involvement (2), suggesting that such autoantibodies may be of clinical relevance. Nonetheless, the effect of these antibodies are still a matter for debate (3), so that it remains uncertain whether they are pathogenic agents or merely markers of vascular damage. The former hypothesis is likely to be correct inasmuch as it has been well established that they can induce cell activation (4) and apoptosis (5).

Furthermore, treatment of EC with cytokines enhances the expression of surface components, most notably adhesion molecules. Activation of EC has been demonstrated to be relevant in the interaction between AECA and the endothelial surface. In Kawasaki's disease, binding of AECA resulted in cytotoxic activity towards interleukin (IL)-1, interferon (IFN)- γ and tumor necrosis factor (TNF)- α -stimulated endothelium, but not towards unstimulated EC (6). The reverse situation has been encountered in haemolytic uraemic syndrome, where the cytotoxic activity of AECA operated on unstimulated EC but disappeared following activation of the cells (7).

Numerous methods have been developed to determine AECA, including fluorescence-activated cell sorter analysis (FACS), Western blotting (WB), and solid phase immunoassays such as ELISA and RIA. Given the marked diversity of the results, it appears clear that there is a crucial need for standardisation (8). However, comparative studies of different methods to evaluate the same samples are rare (9, 10). Each of these methods has certain limitations, but different ELISA using fixed cells as substrate have thus far been selected in most studies.

For the present study, it was decided to compare the reactivity of AECA in SLE, WG, MPA and CS using three tests: ELISA, FACS and WB. Then the interaction of AECA with activated EC was estimated by comparison with their reactivity on resting endothelium by the same three methods. It was found that distinct AECA groups were detectable, depending on the technique.

Material and methods

Source of human sera

Sera were obtained from patients with WG (n=30), SLE (n=17), CS (n=12) and MPA (n=12). All of these patients fulfilled the respective criteria for the diseases. For the ELISA, 81 AECA-negative sera selected from among those sent to the laboratory on a routine basis were taken as controls to set the cutoff level. None of these were from patients with diseases acknowledged to be associated with AECA (1), or anti-mitochondrial and anti-lysosome autoantibodies. Thirteen different normal sera obtained from the laboratory staff served as controls for the FACS analysis and Western blot. All sera were stored at -70° C until used. The controls were matched for sex and age to the vasculitis patients.

Cell culture

EA.hy 926 hybrid cells (kindly donated by CJS Edgell, University of North Carolina, Chapel Hill, NC) were grown in DMEM (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (BioWhittaker, Emerainville, France), 50 g/ml gentamycin (Sanofi-Winthrop, Gilly, France), 2 mM L-glutamine (Eurobio, Les Ulis, France) and HAT consisting of 100 M hypoxanthine, 0.4 M aminopterin, and 16 M thymidine (Sigma Chemical Co., St Louis, MO.). Using a large panel of monoclonal antibodies, we have previously shown (11) that such a cell line exhibited the whole phenotype of human umbilical vein endothelial cells (HUVEC), but we cannot ensure that putative target antigens for AECA might be missing. We failed, however, to find any differences between EA.hy926 cells and HUVEC in pilot experiments in a pre-

vious study focusing on AECA in systemic scleroderma (12). IL-1-activated EC were obtained by incubating EA.hy926 for 6 hours in the same medium supplemented with 50 U/ml IL-1 (Genzyme, Cambridge, MA). LPS (a gift from S Chesne, Grenoble, France), TNF- and IFN- (both from Genzyme) were also tested in preliminary experiments.

ELISA for the detection of AECA

Cultured EA.hy926 were harvested using Trypsin-EDTA (Eurobio) and seeded onto 96-well microtiter plates (Nunc, Roskilde, Denmark) at a density of 10^4 cells per well. These were allowed to grow until confluence, washed and fixed with 100% ethanol on ice for 5 minutes, or with 0.1% glutaraldehyde for 10 minutes at 4°C. We have previously reported (13) that, due to the unpredictable loss of cells, it was absolutely impossible to use unfixed EC in the test. Using 2% bovine serum albumin, the plates were blocked for 2 hours at 37°C. After three washes with phosphate buffer saline (PBS), 100 μ l of serum diluted 1/100 in PBS supplemented with 10% FCS was added in triplicate to each plate for 1 hour. The addition of FCS avoided binding of heterophile antibodies to the plate (13, and Meroni, personal communication). Each plate had a blank value defined as a well without cells (blocked with PBS-10% FCS). Incubation with horseradish peroxidase (HRP)-conjugated rabbit F(ab')₂ anti-human IgG 1/4,000 (Dako, Copenhagen, Denmark) was then carried out for another hour followed by incubation with O-phenylenediamine (0.2 g/l in 0.05 M PBS, pH 5; 0.5 mM H₂O₂). The absorbance was measured at 492 nm and the optical density (OD) of control wells (without cells) was subtracted from that of the wells with EA.hy926 to account for non-specific binding. The mean + 2 SD of 81 control sera was taken as the threshold for positivity. In a previous study (12), all the sera were absorbed with A549/8 cells, the epithelial parent of the hybrid cell line before evaluation, but this did not cause any changes in the results.

Flow cytometry

EC were trypsinized and washed twice with ice cold PBS. There was a 30-minute incubation of 100 μ l of sera, diluted 1/100 in PBS, with 2×10^5 cells at 4°C. The cells were then incubated with FITC-conjugated mouse F(ab')₂ anti-human IgG 1/20 (Dako) for 30 minutes. An unconjugated mouse monoclonal anti-E selectin antibody was included (Beckman Coulter, France, Paris, France) in the experiment to evaluate the effect of IL-1 on the cells. The monoclonal antibody binding was developed with a FITC-conjugated goat F(ab')₂ anti-mouse IgG antibody, diluted 1/10 (Dako). Results were analysed on an ELITE flow cytometer (Beckman Coulter). A serum was considered as positive when the percentage of positive cells was greater than the mean + 2 SD of 13 normal sera selected for these experiments. The mean fluorescence intensities (MFI) were also recorded for comparison with the normal controls.

Membrane preparation

Membrane extracts were prepared using a method described previously (14). Cells were scraped off with a police rubber man and resuspended in a homogenisation buffer (1 M sucrose, 100 mM Tris-HCl, 100 mM EDTA, 0.2 mM PMSF, 100 mM KCl, 50 mM MgCl₂, 1 M leupeptine, 1 M pepstatine, 1 M aprotinine). Cells were then submitted to two cycles of freezing/thawing in liquid nitrogen and sonicated (three times, each for 15 seconds). Nuclei were removed by centrifugation at 1,000 g for 10 minutes. Given that mitochondria and lysosomes were pelleted together with the plasma membranes, special attention was paid to exclude sera found to be positive for the related autoantibodies in the standard indirect immunofluorescence tests.

Supernatants were collected and ultracentrifuged at 100,000 g for 30 minutes. The pellet containing the membrane-enriched extracts was then resuspended in solubilisation buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT) and left rocking overnight at 4°C. Finally, solubilised proteins were

recovered by centrifugation at 11,000 g. Proteins were loaded on a 5-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gradient and blotted onto a nitrocellulose membrane. The protein concentration was determined by the MicroBCA protein assay kit (Pierce, Rockford, IL).

Immunoblotting

The membranes were blocked for 2 hours with PBS-2% non-fat milk 0.05% Tween 20 and incubated overnight with sera diluted 1/50 in PBS-1% non-fat milk-0.05% Tween 20. Biotin-conjugated goat F(ab')₂ anti-human IgG, diluted 1/1,000 (Beckman Coulter), was dispensed onto the membranes with a second-layer of HRP-conjugated streptavidin, diluted 1/300 (Amersham Pharmacia Biotech, Orsay, France). The membranes were developed by diaminobenzidine with H₂O₂ in 50 mM Tris, pH 7.6.

Densitometric analysis

The pattern of reactivity of each serum was determined using a BioRad model G-700 imaging densitometer and Molecular Analyst®/PC software (V. 1.4.1). The molecular weight of the migrated proteins was evaluated, and the OD of those recognized by AECA was recorded. Normal sera were added to each blot. A patient's serum was regarded as positive either when an enhanced intensity compared with controls (over 0.1 in OD) was observed for a given peak, or when an additional peak was found (in this respect, the cut-off value was arbitrarily set at 0.1).

Statistical analysis

All the results quoted below are arithmetic means + SEM. The net OD results of the ELISA were compared using the Mann-Whitney test, rather than the numbers of positive.

Results

AECA detected by ELISA

We measured the amount of AECA in human sera in a cell ELISA on fixed cultured EC. The cutoff OD for positivity was given by the mean + 2 SD of values from 81 control sera, i.e., 0.360. Using this assay, we analysed AECA

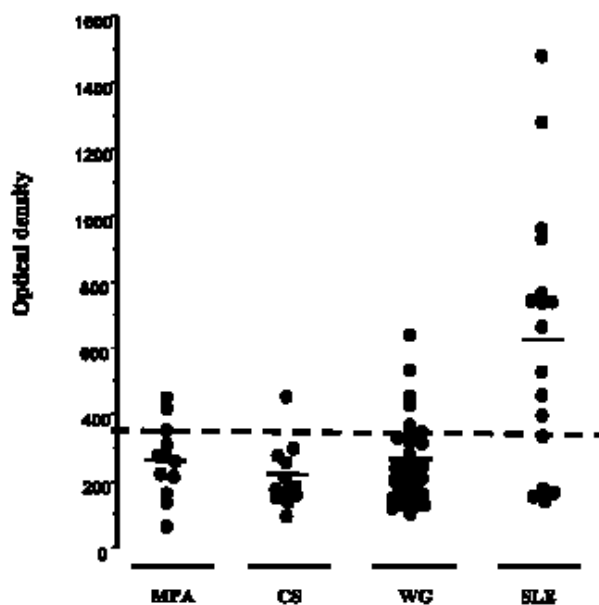


Fig. 1. Anti-endothelial cell IgG in serum from patients with vasculitis by ELISA. The dashed line indicates the cut-off level (mean + 2 SD of 81 normal sera). MPA: micropolyarteritis, CS: Churg and Strauss syndrome, WG: Wegener’s granulomatosis, SLE: systemic lupus erythematosus.

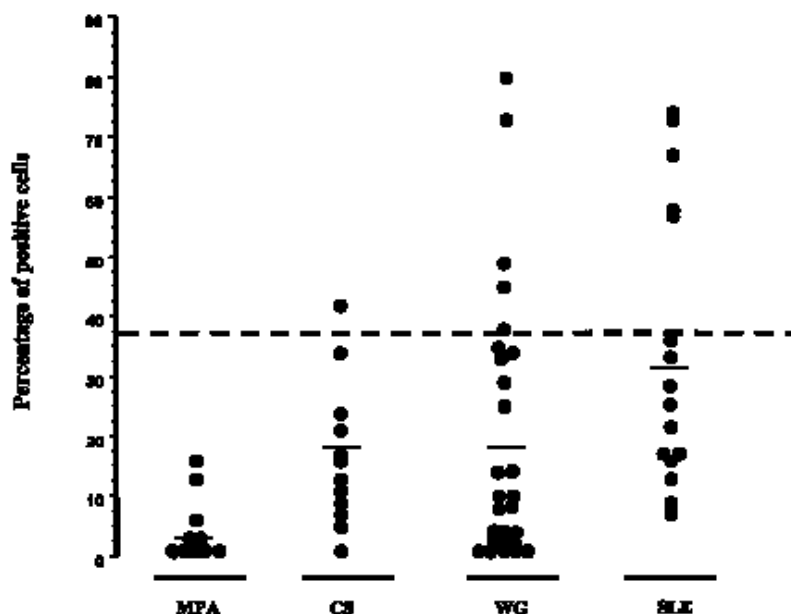


Fig. 2. FACS analysis of anti-endothelial cell IgG in serum from patients with vasculitis. The threshold for positivity is indicated by the dashed line which is the mean value + 2SD of eight normal controls. See figure 1 for abbreviations.

Table I. Reactivity of vasculitis sera on non-activated cells with three different methods. See legend to figure 1 for abbreviations.

Diseases	ELISA		Number of positive (%)		Western-blot	
			FACS			
MPA (n=12)	3	(25)	0		5	(42)
CS (n=12)	1	(8)	1	(8)	3	(25)
WG (n=30)	5	(17)	4	(13)	17	(57)
SLE (n=17)	21	(71)	5	(29)	12	(71)

reactivity in sera from 17 SLE, 12 MPA, 12 CS and 30 WG patients (Fig. 1). Significantly more SLE patients were found to be positive (12 out of 17 patients, 71%) than MPA (3 out of 12 patients, 25%), CS (1 out of 12 patients, 8%) or WG patients (5 out of 30, 17%), and the OD were significantly higher, compared with the controls (Mann-Whitney test, $p < 0.01$).

AECA detected by FACS

The FACS analysis allowed us to ascertain whether AECA were directed against intracellular targets rather than membrane-constitutive or membrane-bound molecules. A serum was considered to be positive when the percentage of positive cells was higher than the mean \pm 2 SD of 13 normal sera included in this assay (Fig. 2). For the FACS analysis, the cut-off level was fixed at 38% of positive cells. Of note is that the vast majority of sera reacted weakly with EC by FACS compared to ELISA. Whereas 12 of 17 SLE sera were positive in the ELISA, only 5 sera showed AECA against unfixed EC by FACS. No reactivity was observed in the MPA group, only one serum was positive in the CS group, and 4 were positive in the WG group compared to 3, 1 and 5 sera from these groups respectively on the ELISA (Table I). Overall, the percentages of cells stained by the 10 positive sera were $55.7 \pm 3.2\%$ ($p < 0.01$, compared with the controls, $14.5 \pm 2.3\%$), and the MFIs reached $18.2 \pm 1.1\%$, ($p < 0.01$, compared with the controls, 9.1 ± 0.5).

AECA detected by immunoblotting of membrane resting EC

The pattern of reactivity of each serum was evaluated by densitometry. Compared to that of 13 normal sera, positivity was defined according to either one of two criteria. The first criterion was when control and patient sera recognized the same band; this band had to be more marked, with an extra OD over 0.1 in the patient, compared with the control. Alternatively, a patient serum was considered to be positive when it showed reactivity with an original band. In fact, binding of IgG to enriched membrane preparations reacted

against multiple bands with molecular weights ranging from 200 to 24 kDa. AECA was detected in sera from 12 of 17 SLE patients with 19 bands in contrast to 7 bands in the controls. Seventeen of 30 WG patients (57%), 3 of 12 CS patients (25%) and 5 of 12 MPA patients (42%) had reactivity with 16, 8 and 13 bands respectively.

Some bands were recognized by some sera but not by others. Control sera gave a background consisting of seven bands (36, 40, 49, 52, 65, 72 and 85 kDa). Some AECA patients recognized the same proteins, especially those of 52, 65 and 72 kDa in molecular weight. Eleven sera from patients with SLE reacted with at least one original band among the 24, 37, 42, 44, 70, 71, 90 and 120 kDa proteins. Two bands (24 and 37 kDa) were recognized by 5 SLE sera. Fifteen WG out of 30 showed a reactivity with original bands (68, 70, 77, 80 and 120 kDa), four MPA with 24, 68, 70, 80 and 120 kDa, and three CS with 44, 68 and 120 kDa. In all of these diseases, AECA recognized some antigens with abnormally strong intensity compared to normal sera.

Densitometric profiles of reactivity of a control serum and a SLE serum are presented in Figure 3. SLE sera offered a greater reactivity with antigens of identical molecular weight (49, 52, 65 and 85 kDa) compared to the normal serum, and also bound to original antigens (37 and 120 kDa).

Comparison of reactivity measured by three methods

In comparison with fixed cells in the ELISA, reactivity with non-fixed cells analysed by FACS was reduced. The frequency of positive results was even higher in the WB than in the ELISA (Table I). Some AECA detected by ELISA on fixed cells seemed to be directed towards intra-cytoplasmic antigens, particularly those from SLE which were positive in the ELISA and negative by FACS (42%). Similarly, antigens revealed by Western blotting were different from those detected by ELISA and FACS. For example, 50% of the WG sera were negative in ELISA and FACS, but positive on the blots (Table II).

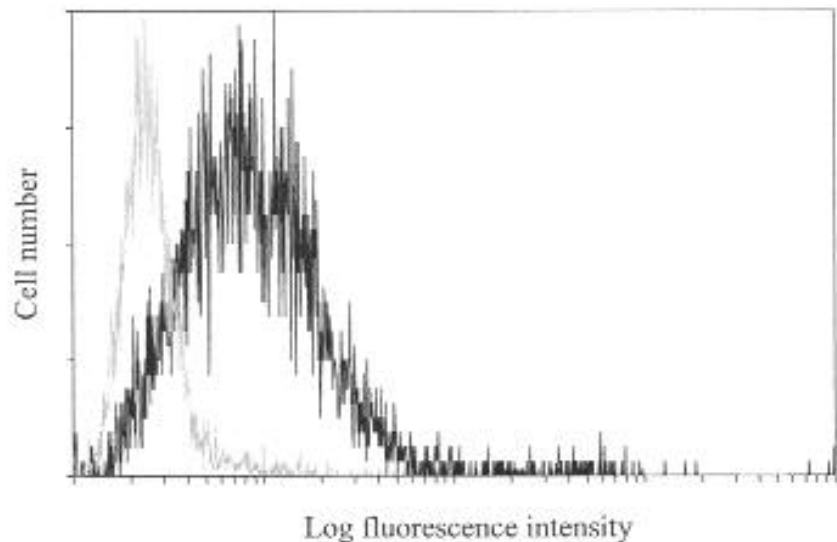


Fig. 3. Binding of IgG from a patient with WG (solid histogram) and a control serum (faint histogram) to endothelial cell by FACS.

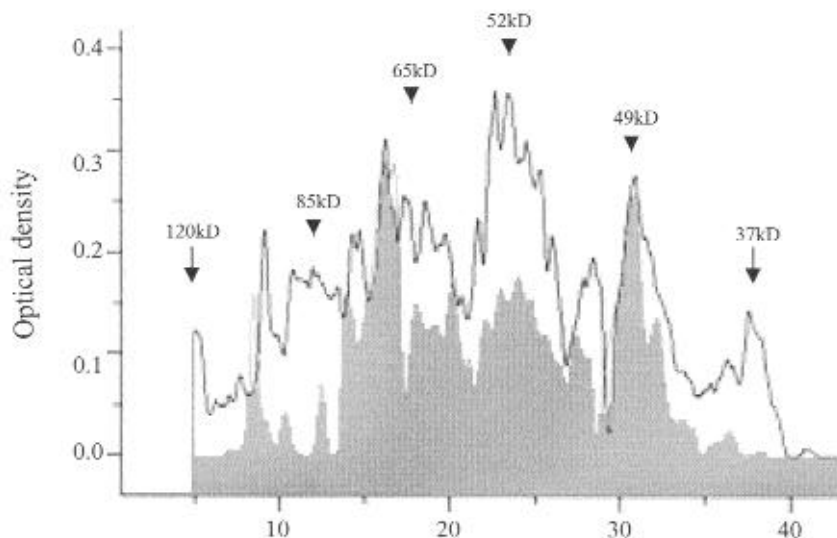


Fig. 4. Densitometric profile of reactivity of AECA IgG from a patient with systemic lupus erythematosus (white area) compared to that of a control normal serum (grey area). Patients' antibodies recognize antigens in addition to natural antibodies (arrows). Some antigens are identified by the normal serum, but more intensively by the pathologic serum (arrowheads).

Effects of activation on the binding of AECA IgG to EC

Stimulation of EC by IL-1 was accounted for by the membrane expression of E-selectin (Fig. 5). After activation 89% of the cells expressed E selectin compared to 2% of the resting cells, and the MFI was raised to 22.8 compared to 14.9 in the resting cells. VCAM-1 and ICAM-1 were also increased in density, while there was a downregulation of thrombomodulin (15) indicating that the cells were activated (16).

The ELISA and FACS reactivity of AECA from all the patients investigat-

ed was not significantly modified when measured using IL-1-stimulated EC compared with unstimulated EC (data not shown). However, differences were observed in the binding pattern of serum AECA to blotted antigens prepared from IL-1-stimulated EC when compared to antigens prepared from unstimulated EC (Table III). There appeared to be three situations: either binding of AECA on activated EC was increased as established by densitometric analysis; or novel surface antigens on EC were recognized; or both previous situations co-existed.

Table II. Comparison of the results obtained using three different methods. See legend to Figure 1 for abbreviations.

ELISA	FACS	MPA (n=12)	CS (n=12)	WG (n=30)	SLE (n=17)
+	+	0	0	2	3
+	-	3	1	3	9
-	+	0	1	2	2
-	-	9	10	23	3

ELISA	W. blot	MPA (n=12)	CS (n=12)	WG (n=30)	SLE (n=17)
+	+	0	0	3	9
+	-	3	1	2	3
-	+	5	3	14	3
-	-	4	8	11	2

ELISA	W. blot	MPA (n=12)	CS (n=12)	WG (n=30)	SLE (n=17)
+	+	0	0	2	5
+	-	0	1	2	0
-	+	5	3	15	7
-	-	7	8	11	5

Table III. Effects of activation on the pattern of reactivity of vasculitis sera by Western blot. Three cases are observed: (a) the binding can increase upon activation, (b) additional antigens can be detected only on activated cells and finally, (c) both situations may coexist.

Diseases	Raised staining (a)		Original bands (b)		Raised and original bands (c)	
MPA (n = 12)	1	(8)	5	(42)	2	(17)
CS (n = 12)	0		2	(17)	0	
WG (n = 30)	1	(3)	8	(27)	7	(23)
SLE (n = 17)	3	(18)	4	(23)	5	(29)

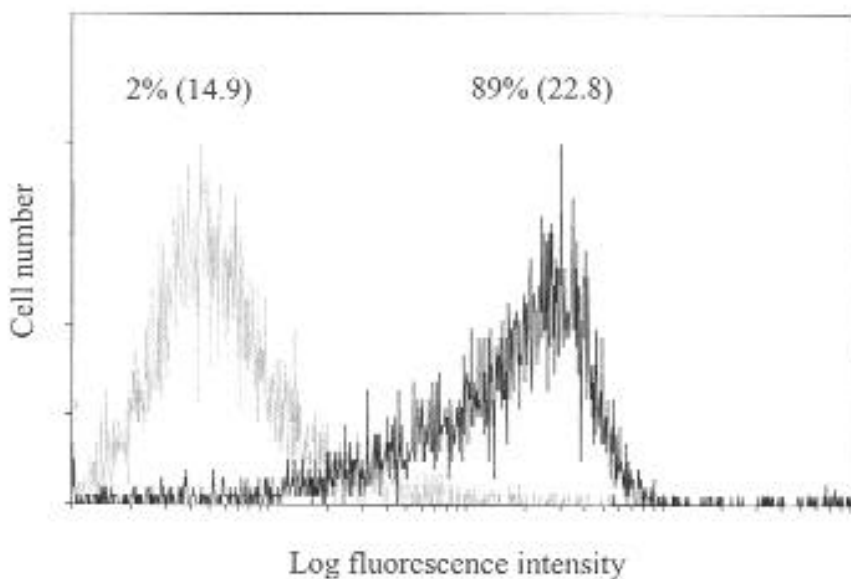


Fig. 5. Activation of endothelial cells by IL-1. Non-activated cells do not express E-selectin marker (faint histogram), whereas IL-1-activated cells do (solid histogram). Values represent the percentage of positive cells and the mean intensity of fluorescence.

At a glance, there was no convincing disease-specific pattern of reactivity. When a band was ascribed to serum from a patient with a given disease on activated EC, the same band could be identified by a serum from a patient with another disease. It must, however, be highlighted that five MPA (42%) sera identified a 100 kDa band and eight WG sera (27%) bound to a 44 kDa band. In the majority of sera, the intensity of the binding pattern of AECA to blotted antigens was raised, particularly in the range of 60 to 80 kDa in MPA and WG but also in SLE and around 24 kDa in SLE. A representative example of MPA is shown in Figure 4. In return, activation did not generate the disappearance of reactivity, except for one CS serum of which AECA recognized one band, but only on resting cells.

Discussion

A striking settlement on AECA is the variability of their prevalence in various disease. For example (1, 8), they were reported in 15 to 88% of the cases in SLE, and in 30% to 80% of the cases in WG. These discrepancies are, most probably, due to the lack of standardisation of the cell ELISA and, presumably, to methodological pitfalls (13). Such artefactual observations cast doubt on some results, including ours. In the present study, 70% of SLE sera which is in line with reports (17). In contrast, only 17% of WG sera were found positive in the cell ELISA. Although 50% of the WG sera were scored positive by WB, this reduction may be explained by our technique developed to obviate false positivity due to natural antibodies against animal serum proteins (13), inasmuch as all our patients including those with WG, were in an active phase of their disease. A number of methods have been compared with each other using positive sera selected by ELISA (9, 10), but ELISA-negative sera were not considered in these two studies. Both positive and negative sera were evaluated in our experiments to rule out any bias. We observed that a number of sera were negative in the ELISA but turned out to be positive using another test. Interest-

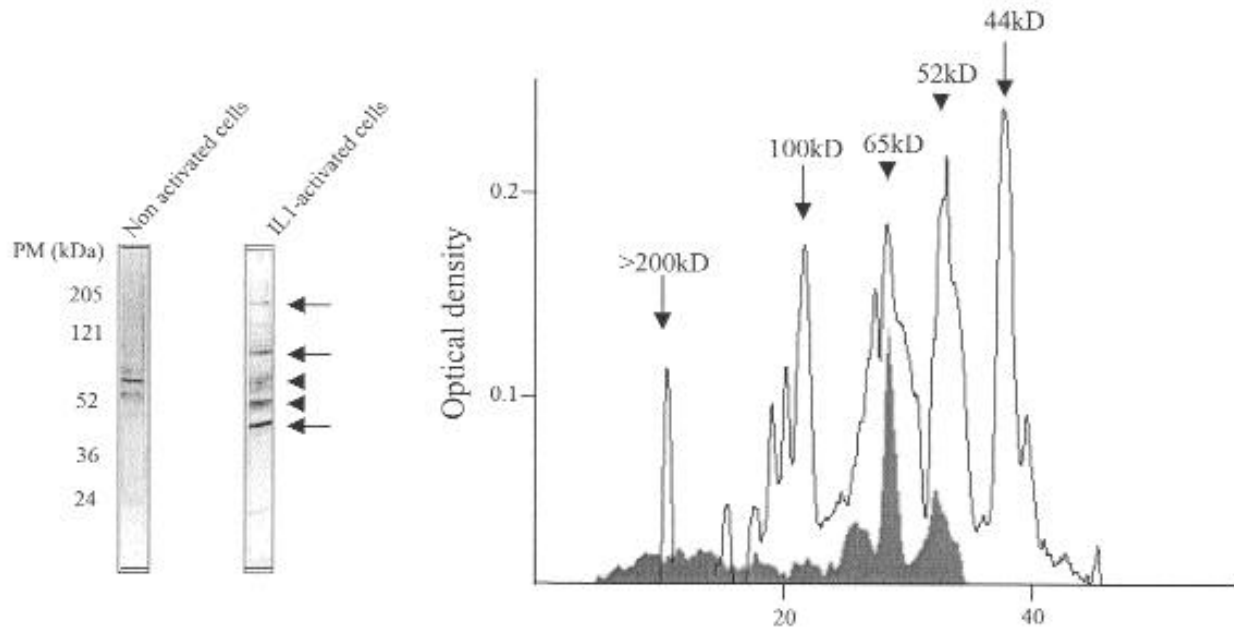


Fig. 6. Pattern of reactivity of IgG in the serum of a micropolyarteritis patient on membrane-enriched extract from non-activated (black area) and IL-1-activated endothelial cells (white area) Arrows indicate additional peaks, arrowheads point to bands that are recognized by the serum on both cell membrane extracts but with a stronger intensity on IL-1 activated cells (A.U: arbitrary units).

ingly the loss of reactivity from ELISA to FACS were also described by Westphal *et al.* (9). Fixed cells used in ELISA are slightly permeabilized and for that reason we detected AECA against cytoplasmic and membrane antigens, whereas by FACS analysis performed on unfixed cells the reactivity was restricted to membrane antigens. It may be argued that other fixation procedures do not permeabilize the cells as much. We used methanol or glutaraldehyde, since it has been reported by Del Papa *et al.* that EC membrane alteration induced by paraformaldehyde non-specifically enhances the binding of affinity-purified AECA preparations (18). Alternatively, trypsin used to detach EC before FACS analysis might have altered surface antigens. Antibody cell binding followed by fixation might be more appropriate and evaluation of this method is currently underway.

Among sera which were negative by ELISA or FACS analysis, 50% WG, 40% MPA, 20% CS and 40% SLE became positive when studied by WB. Clearly, a negative ELISA does not imply that AECA are absent. Various hypotheses may account for these differences, such as: (i) the denaturing

conditions used in the immunoblotting experiment may expose cryptic epitopes recognised by AECA, while conformational epitopes are eliminated; or (ii) staining amplification by the biotin/streptavidin system with immunoblot may be more sensitive than signals obtained by ELISA or FACS analysis. We have detected nineteen bands with SLE sera instead of fifty nine bands found by others (15).

Intriguingly, the control sera produced more signals than previously obtained in several studies (17, 19). At least seven bands were detected with the control group. These antigens were equally recognised by AECA from the patients' sera but with greater intensity. However, Kazatchkine *et al.* have described natural AECA directed against EC or another type of cells (20, 21). Comparable patterns were obtained in the present densitometric analysis.

The amplified methods and antigenic concentrations used in our studies could explain the increase in staining obtained with the control as well as patient sera. Some AECA reacted with original bands, but no specific profile appeared to be associated with any one pathology. Two particular proteins (24 kDa and 37 kDa) were detected with

SLE sera which might show promise as an indicator for the prognosis. Several groups have noticed a correlation between a 37 kDa protein and nephritis or disease activity in SLE (15, 20). A certain proportion of WG sera showed reactivity against proteins ranging from 60 kDa to 80 kDa, as previously described (22). Although this reactivity was not exquisitely disease-specific, judging by the similar patterns obtained with some of the MPA or CS sera, it might be of use in the diagnosis. Only the CS group expressed a low titer of AECA by all three methods. Due to this low reactivity, one may hypothesize that such AECA are secondary to vascular damage, rather than causative. If AECA played an active role of the pathogenicity of the disease, one might expect much higher prevalence.

Our studies of the influence of EC activation on AECA binding with the ELISA and FACS methods did not confirm the spectacular increased binding previously described in Kawasaki's disease (7). However, with the immunoblotting method, an increased staining of proteins between 60 and 80 kDa was found in SLE sera. The binding of AECA protein after EC activation on 100 kDa and 44 kDa proteins for MPA

and WG sera, respectively, needs to be confirmed by further experiments. Again, the importance of the method employed must be emphasized, because van der Zee *et al.* reported an increased binding of SLE sera on stimulated EC in ELISA but failed to confirm these results in the immunoblot (20). There is every likelihood that these discrepancies might be due to differences between the antigenic preparations in the three methods. Caution must therefore be exercised when interpreting the results of any of the three tests. None may be used to screen the sera and ideally all three methods should be applied.

Further work needs to be done to characterize in more detail the target antigens of AECA. A study involving two-dimensional gel electrophoresis followed by peptide mass fingerprinting analysis is currently in progress in our laboratory, in order to isolate membrane-bound antigens from stimulated and non-stimulated EC. This strategy may be helpful to further define the effect of autoantibodies in vasculitis diseases. Similar conclusions have been drawn by Faulk *et al.* who utilized ELISA, FACS and immunoblot to detect AECA in 68 cardiac allograft recipients (10).

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