Detection of Antiendothelial Cell Antibodies by an Enzyme-Linked Immunosorbent Assay Using Antigens from Cell Lysate: Minimal Interference with Antinuclear Antibodies and Rheumatoid Factors

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The objective of the present work was to set up a routine test adapted to screening for antiendothelial cell antibodies (AECAs) in serum samples with minimal interference from antinuclear antibodies (ANAs) or rheumatoid factors (RFs). We compared the titers of AECAs titrated following two enzyme-linked immunosorbent assays (ELISAs): (i) an ELISA with ethanol-fixed EA.hy926 monolayers as the antigenic substrate and (ii) an ELISA with nucleus-depleted lysates prepared from EA.hy926 cells and normalized for protein (1.0 to 1.7 mg/ml) and DNA (≤0.1 μg/ml) contents as a surrogate substrate (postnuclear supernatant ELISA [PNS-ELISA]). The AECA titers in 51 serum samples, including 28 samples containing ANAs, were compared. A significantly positive correlation (r = 0.77; P < 0.001) between the two series was shown only for the ANA-negative serum samples. Conversely, ANAs or RFs in samples were shown not to interfere in tests for AECAs by the PNS-ELISA. AECAs recognize their antigenic targets in postnuclear supernatants, which is representative of the endothelial antigenic content, with improvement of the reliability of the assay, a prerequisite to application of the assay for their evaluation in clinical practice.

Antiendothelial cell antibodies (AECAs), a heterogeneous group of autoantibodies, are associated with several diseases characterized by immune-mediated vascular damage (for reviews, see references 2 and 13), including systemic lupus erythematosus (19), systemic sclerosis (17), Wegener’s granulomatosis (6), and rheumatoid arthritis complicated by vasculitis (7). Even if they recognize poorly characterized targets, they may be valuable as markers of disease activity, with a possible role in the pathophysiology of associated diseases, especially by inducing endothelial cell activation or apoptosis (2, 3, 12, 23). The presence of AECAs in the sera of some groups of patients may be an important etiopathogenic factor in the vasculopathy associated with the disorders mentioned above and classified as proposed by Praprotnik et al. (14). The association of AECAs with endothelial injury in the course of these diseases prompted us to develop assays for these antibodies in clinical practice, which in some cases requires histopathological examination of affected organs for confirmation.

Most assays usually used for the detection of AECAs involve human umbilical vein endothelial cells or endothelial cell lines seeded in microtiter plates for subsequent testing by enzyme-linked immunosorbent assay (ELISA) or assays in which AECAs are detected by immunofluorescence. Spurious increases in AECA titers may occur, e.g., due to anti-DNA autoantibodies, depending on an important cross-reactivity against endothelial cells (4); anti-heparan sulfate antibodies (16); or heterophile antibodies to the bovine serum proteins involved in the assay (18). One of the main problems in this field is the lack of agreement on a standardized method for detection of AECAs, with subsequent difficulty with interlaboratory comparisons (13, 24). In addition, in rheumatoid arthritis and Felty’s syndrome, rheumatoid factor (RF) has been shown to increase nonspecific immunoglobulin binding to endothelial cells, with subsequent pitfalls in assays with RF-containing sera developed with endothelial cells (15).

In order to minimize these false-positive interferences and to propose a routine simple screening test for the detection of AECAs in patients with autoimmune vascular disorders, we developed a highly reproducible ELISA using a normalized cell lysate preparation. The detection of AECAs by this assay was found to be independent of the presence of antibodies with unrelated specificities, such as antinuclear antibodies (ANAs), antiactin antibodies, and RFs.

MATERIALS AND METHODS

Patient and control samples. Serum samples were collected from patients with various non-organ-specific autoimmune disorders (including connective tissue disease, vasculitis, antiphospholipid syndrome, or viral infection) and were selected because their AECA concentrations covered a wide range, from 0.02 to 1.17 absorbance units (reference value, 0.2 absorbance units), as established by the cyto-ELISA described below. Sera from 40 healthy blood donors matched for age with the patients served as controls. All samples were kept frozen at −80°C until use.
Endothelial cells and preparation of cell lysates. The human endothelial hybrid cell line EA.hy926 (a kind gift from C.-J. S. Edgell, University of North Carolina, Chapel Hill), obtained by fusing human umbilical vein endothelial cells with the human lung carcinoma cell line A549, was cultured in fetal calf serum (FCS, Gibco; Cergy-Pontoise, France) and extractable nuclear antigens (ENA) specificities were established by both ELISA (Euroimmun-Bio/Advance or Pharmacia, St. Quentin-Yvelines, France) and dot blotting (Inolia-Ingen, Rungis, France). Anti-double-stranded DNA (anti-dDNA) antibodies were titrated by ELISA (The Binding Site, Saint Egrève, France). Anti-human IgG RFs (IgM isotype; reference titers, 30) were measured as described by Gioud-Paquet et al. (5) using an international standard (Bio-Rad Clinical Diagnostics, Marnes la Coquette, France).

Adsortion of AECA and ANA activities. A total of 200 μl of serum (diluted 1:100 in saturation buffer) was incubated for 15 min on ice with either ethanol-fixed EA.hy926 cells (confluent cell layers were prepared as described above), 5 μg of bovine serum albumin (BSA) as a negative control, or 2 μg of PNS antigens. These incubations were repeated four times, and then the A_{450} were measured for determination of the AECA (PNS-ELISA) and anti-DNA antibody titers.

RESULTS

Characterization of PNS preparations. In order to develop a routine test for the detection of AECAs, we considered it to be of importance that the endothelial cell preparation involved as antigen in the assay be representative of the steady-state antigenic content of endothelial cells for all antigens except the nuclear and cytoskeletal antigens. This was achieved by a short incubation of EA.hy926 cells in the presence of mild detergent, keeping the integrity of the nuclei, according to technical recommendations for experimental development in cell biology (11). The nuclei and cytoskeletons were discarded in the pellets, and PNS was harvested as described in Material and Methods, which ensured that the antigen-normalized solution had protein concentrations ranging from 1.0 to 1.7 mg/ml and DNA concentrations less than 0.1 μg/ml.

Dose-response curves, cutoff assignment, and inter- and intr-assay variations. The PNS prepared as described above was distributed in flat-bottom wells of microtiter plates in order to perform the PNS-ELISA, as described above in Material and Methods. To establish the working dilution of the serum samples, the dose-response curve for a positive control sample found to be positive by the cyto-ELISA, as described previously (18), exhibited a dose-dependent response in the PNS-ELISA, reaching a maximum A_{450} of 0.945 ± 0.035. A serum sample from a healthy donor found to be negative, as described above, had an A_{450} of 0.215 ± 0.018; the values for the blank wells for these positive and negative controls were 0.235 and 0.185, respectively. A representative example of the titration curves for the positive serum sample and the negative control serum sample from a healthy individual are shown in Fig. 1. The 1:100 dilution was used as the working dilution for all subsequent assays.

To establish the cutoff value of the test, 72 serum samples from healthy donors were analyzed, and after subtraction of the values for the blank wells, A_{450} values ranged from 0.006 to 0.165 (mean ± 1 SD, 0.070 ± 0.040). The cutoff value was calculated by taking the mean ± 3 SDs, i.e., 0.190; this was assumed to give an index value of 100. As suggested by Rosebaum et al. (19), the index was calculated by the equation 100 X (S - A)/(C - A), where S is the A_{450} value for the sample and A and C are the A_{450} values for the negative control and the control used to establish the cutoff value, respectively.

ELISAs for AECA. Two assays were compared for the measurement of AECAs: an already established cyto-ELISA, in which endothelial cell monolayers constituted the target for antibodies, and a PNS-ELISA, developed with a normalized antigen preparation as a constant source of antigen. In both assays, the mean + 3 standard deviations (SDs) for 72 serum samples from healthy individuals was taken as the cutoff point of the test.

Ethanol-fixed EA.hy926 cells were used in the cyto-ELISA, as described previously (18). Briefly, EA.hy926 cells were plated (10⁴ cells/well in FCS medium) in flat-bottom microtiter plates (Nunc, Kastrup, Denmark). Confluent cell layers were washed with PBS buffer and fixed with 100 μl of absolute ethanol for 5 min at 4°C. After three washings in PBS buffer, the plates were blocked with PBS buffer containing 1% (wt/vol) bovine serum albumin and serum samples were then successively exposed to 100 μl of 1:100 dilution of a positive serum sample as a positive reference and a 1:100 dilution of a negative serum sample as a negative reference, followed by incubation with 0.2 g of Ethanol-adsorbed EA.hy926 cells (confluent cell layers were prepared as described above), 5 μl of PBS buffer containing 1% (wt/vol) bovine serum albumin and were then successively exposed to 100 μl of peroxidase-conjugated rabbit F(ab')₂ anti-human immunoglobulin G (IgG), IgM (diluted 1:4,000) and IgA (diluted 1:2,000) antibodies (Dako, Trappes, France), followed by incubation with 0.2 g of o-phenylenediamine per liter, 0.5 ml of H₂O₂ per liter, and 0.05 M sodium phosphate (pH 5.0). For each serum sample, the mean absorbance at 492 nm (A_{492}) for blank wells was subtracted from the mean values for the wells with EA.hy926 cells to account for nonspecific binding. Data were expressed in absorbance units (A_{492} for sample wells – A_{492} for negative control wells).

The PNS-ELISA was performed as follows. Each well of half of the microtiter plates (MaxiSorp; Nunc) was coated overnight at 4°C with 100 μl of H₂CO₃ (pH 9.6) (antigen-coated wells), and each well of the other half of the plates was coated for 15 min at 37°C with antigen-normalized solution (PNS), and the PNS antigen preparation was thus suitable for use in the PNS-ELISA. It was stored at −80°C until use. All chemicals were of analytical grade.

ELISAs for ANA. Other autoantibodies. ANAs and antinuclear antibodies were detected and quantified by titration on Hep-2 cells (reference titers, 80; Euroimmun-Bio/Advance, Emernainville, France), and extractable nuclear antigens (ENA) specificities were established by both ELISA (Euroimmun-Bio/Advance or Pharmacia, St. Quentin-Yvelines, France) and dot blotting (Inolia-Ingen, Rungis, France). Anti-double-stranded DNA (anti-dDNA) antibodies were titrated by ELISA (The Binding Site, Saint Egrève, France). Anti-human IgG RFs (IgM isotype; reference titers, 30) were measured as described by Gioud-Paquet et al. (5) using an international standard (Bio-Rad Clinical Diagnostics, Marnes la Coquette, France).
Finally, the samples were classified as having negative (<100), medium-positive (100 to 250), and high-positive (>250) AECA titers.

The interassay variation was determined by calculating parameters for the descriptive statistics for the samples. Twenty serum samples were tested on different days, with AECA titers being above the reference value (medium to high titers) for 11 samples and below the reference value (low titers) for 9 samples. The corresponding coefficients of variation were from 1.1 to 18.9% for samples with medium to high AECA titers and from 8.2 to 22.6% for samples with low AECA titers (Table 1). The intra-assay variation was similarly determined with nine samples (four with medium to high titers and five with low titers), with coefficients of variation from 2.9 to 5.4% for samples with high AECA titers and 3.2 to 8.9% for samples with low titers (data not shown).

Comparison of AECA titers by cyto-ELISA and PNS-ELISA.

(i) Interference of ANA. Fifty-one serum samples submitted for routine testing for AECAs, including 28 serum samples that were ANA positive (ANA titers, >160) and that were chosen because of their ranges of AECA titers, were assayed by both the cyto-ELISA and the PNS-ELISA. The ANA-positive sera expressed antibodies of diverse specificities (PM-Scl, RNP, U1-RNP, SS-A, SS-B, centromere, PCNA, dsDNA). We first compared the AECA titers in all 51 samples obtained by both assays and found a correlation coefficient (r) of 0.33 (P < 0.02) (Fig. 2), indicating a weak correlation between the methods. We then compared both ELISAs for their abilities to detect AECA titers in the 23 ANA-negative samples and observed an r value of 0.77 (P < 0.001) (Fig. 2), indicating in this case a good correlation of the data between the cyto-ELISA and the PNS-ELISA.

The 28 ANA-positive serum samples appeared to be positive by the cyto-ELISA. A comparison of the AECA and ANA titers led to an r value of 0.65 (P < 0.001) when the values from the cyto-ELISA were used and an r value of 0.20 (P = 0.310) when the values from the PNS-ELISA were used. This suggests an independence between AECA and ANA titers when PNS is used as a substrate but not when fixed EA.hy926 cells are used. When data for all ANA-positive samples were excluded, 16 samples were found to be positive by the PNS-ELISA, whereas 14 were found to be positive by the cyto-ELISA, indicating that the capacities to discriminate between negative and positive samples remained similar for both assays.

We next analyzed data for 50 serum samples with ranges of AECA titers (evaluated by PNS-ELISA) and ANA titers. We observed a nonsignificant r value of 0.13 between the AECA and the ANA titers (P = 0.38) (Table 2), indicating the absence of a correlation between the AECA (PNS-ELISA) and ANA titers.

The binding of ANA to the PNS antigen was compared with that to the fixed EA.hy926 cells. An absorption experiment with an ANA-positive sample with anti-DNA specificity was carried out, and the resulting AECA and anti-DNA reactivities were determined. After repeated incubations with fixed EA.hy926 cells, the binding of the antibodies from the sample to the cell antigen resulted in a decrease in the anti-DNA-related A450 that was more than that for the PNS antigen (~30% difference), suggesting that EA.hy926 cells partially removed the ANA activity from the patient’s sample (Fig. 3). Taken together, these results strongly suggest that ANA should not interfere in the PNS-ELISA for AECAs.

(ii) Interference of RFs and antiactin antibodies with AECA titers. RFs are likely to give false-positive results when serum is analyzed by ELISA, in particular, in assays for AECAs with endothelial cells (15). In order to be eligible for use with samples from patients with autoimmune diseases in which RFs are present, the PNS-ELISA for AECAs was developed with the 50 samples containing IgM RFs (up to 5,600 IU/ml) described above. The data are summarized in Table 2.

![Graph](image-url)  
**Fig. 1. Dose-response curves for AECA-positive and -negative control sera.** AECA titers were determined by the PNS-ELISA, as described in Materials and Methods.
ison of the AECA and RF titers gave $r$ values of $-0.20 (P = 0.17)$, indicating an absence of a correlation between the two titers, with a total independence of the AECA and RF titers found in the sera. This result indicates that RFs do not interfere in the PNS-ELISA described for AECAs in this study.

Antiactin-positive sera scored negative for AECAs, suggesting that antiactin antibodies do not interfere in the test (data not shown).

**DISCUSSION**

A number of methods have been developed for the detection of AECAs, including indirect immunofluorescence, radioimmunoassay, Western blotting, assays with cell membranes, and cyto-ELISA. The cell substrates have included endothelial cells derived from different origins, namely, arteries, saphenous or umbilical veins, and endothelial cell lines.

It became important to overcome variations in differences in the tests owing to differences in the endothelial cells (donor variability or cell number), with subsequent high coefficients of variation between the assays. This prompted us to develop an alternative to the cyto-ELISA with an antigenic target of AECAs in a technique that can be used for routine testing after normalization of the antigenic preparation for protein and DNA contents. The advantage of the present ELISA is the fact that the PNS antigen from EA.hy926 cells is a constant and easily accessible source of antigen. Comparison of the results obtained by the two ELISAs, the cyto-ELISA and the PNS-ELISA, shows that they have a good correlation when ANA-negative sera are used, as assessed by Spearman’s sampling correlation coefficient. This is in contradiction to the data reported by Heurkens et al. (8), who used sonicates from cell membranes prepared after scraping of culture monolayers. In that assay the poor correlation observed between the cyto-ELISA and the cell membrane ELISA was assumed to be due to the loss or denaturation of surface antigens. The surrogate preparation used in the present study is representative of the endothelial cell content, including already observed antigenic targets of AECAs expressed as membrane-associated proteins at steady state, as demonstrated by adsorption experiments (8), or expressed within the cytoplasms of endothelial cells (6). Recent experiments attempted to characterize the antigenic targets of AECA in relation to one disease or another; it was concluded that the targets were a mosaic of constitutive antigenic determinants (for a review, see reference 14), for example, the cytoskeletal proteins tubulin and vimentin and the membrane-associated protease collagenase identified by immunoblotting (22), CD36 and a 50-kDa keratin-like protein identified after immunoscreening of a serum sample with a cDNA expression library (1), and an unidentified 70-kDa structure isolated after immunoprecipitation of iodinated membrane protein targets (9). Therefore, it was of importance to consider the PNS as a whole antigenic target and, consequently, to prevent during its preparation the loss of any cell proteins or protein associations by the addition of protease inhibitors in the lysis buffer and by the use of a low temperature during each preparation step.

When we used the cyto-ELISA with fixed endothelial cells, we incidentally noticed high-positive ELISA responses with ANA-positive sera, suggesting that the antibody detected could be related to nuclear antigens and, hence, nonspecific for endothelial cells. This is of particular interest when samples from patients with systemic sclerosis or lupus erythematosus are assayed for AECAs. Indeed, in patients with such systemic disorders, ANAs are frequently found at high titers and AECAs may contribute or may be associated with the pathogenesis and the activity of the disease (3, 10, 20). The experimental procedure that we retained to circumvent the problem.

**FIG. 2.** Correlation between cyto-ELISA and PNS-ELISA. AECA titers in 51 serum samples containing (●) or not containing (△) ANAs at significant titers (titers, ≥160; reference titer, 80) were assayed. The ELISAs were performed with fixed EA.hy926 cell monolayers (cyto-ELISA, horizontal axis; reference value, 0.2 absorbance [A] unit) and a PNS antigen preparation (PNS-ELISA, vertical axis; reference value, 100). According to Spearman’s rank test for comparison of the two series of data, $r$ values were $0.35 (P < 0.02)$ for all data and $0.77 (P < 0.001)$ for data obtained from samples negative for ANAs (open diamonds).
of false-positive results due to ANAs and to properly detect associated genuine AECAs was to discard the nucleus from the PNS antigen preparation. The number of samples which were positive for both AECAs and ANAs was subsequently reduced (Fig. 2), and AECA and ANA titers did not correlate (Table 2). To rule out possible nonspecific reactivity because of intracellular lectins or anticytoplasm antibodies, prior to the ELISA the sample was absorbed in diluent containing PNS prepared from EL4 cells. Moreover, the test result was found to be independent of the presence of RF in the sample, a frequent origin of false-positive results by assays for autoantibodies.

The frequency of detection of AECAs depends on the method developed for their detection. One of the main problems in this field is the lack of agreement on a standardized antigen, with subsequent difficulty with interlaboratory comparisons. The preparation of a PNS from the EA.hy926 cell
line normalized for protein and DNA contents could allow better interlaboratory agreement in the measurement of AECA titers by ELISA and allow the data obtained for the same sera processed by different assays to be compared, as recommended by Youinou et al. (24). In addition, this could contribute to a reduction in the important discrepancies in associations between AECA titers and clinical conditions ascribed to antigen variability (13, 2, 9). Better knowledge of the prevalence of AECA in various autoimmune diseases and improved interpretation of the data could take advantage of this standardization of the endothelial cell antigen and its subsequent use in a routine ELISA.

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