False Positivity in a Cyto-ELISA for Anti-Endothelial Cell Antibodies Caused by Heterophile Antibodies to Bovine Serum Proteins

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Background: ELISAs with fixed endothelial cells or cell lines are widely used screening tests for anti-endothelial cell antibodies (AECAs), but spurious increases occur. We examined interferences by heteroantibodies and means to eliminate them.

Methods: AECAs were measured by ELISA on fixed layers of the human endothelial cell line, EA.hy 926, in a panel of 60 patient serum samples diluted in bovine serum albumin. Heteroantibodies against fetal calf serum (FCS) proteins were demonstrated and characterized in an ELISA—the interference assay—that used FCS-coated plates and Tween 20-containing buffer as blocking agent and sample diluent, as well as by immunoblotting.

Results: In 12 of 60 patient serum samples, spurious increases of AECA titers were produced by endogenous antibodies reacting with FCS proteins from culture medium that were coated onto the solid-phase at the time of cell plating. This mechanism of interference was supported experimentally by exposing extracellular matrix, varying cell density, and incubating wells with FCS alone. The heterophile antibodies were mainly IgG and IgA, and in inhibition experiments, they recognized serum proteins from goat, sheep, and horse. Washing cells free of FCS before plating, or adding FCS (100 mL/L) to the patient sample diluent eliminated spurious signals from all 30 tested sera, but the latter method had practical advantages.

Conclusions: Antibodies against animal serum proteins are a frequent cause of erroneous results in cyto-ELISAs. The interference can be eliminated by simple antibody absorption in FCS-containing dilution buffer.

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Anti-endothelial cell antibodies (AECAs) represent a heterogeneous group of antibodies against poorly characterized targets that have been reported in a variety of inflammatory disorders, and they may be valuable as markers of disease activity (1). Often considered as an epiphenomenon of vascular injury, AECAs may also play a role in the pathophysiology of associated diseases, especially by inducing endothelial cell activation and/or apoptosis (1, 2). Numerous methods have been developed to assay AECAs, including immunofluorescence, immunoblotting, functional assays, and more commonly, solid-phase immunoassays such as ELISAs and RIAs, but parallel studies of the same samples in different tests have been infrequent (3–5). One of the main problems faced in this field is the lack of agreement on a standardized method to detect AECAs, which renders the interlaboratory comparison of the results somewhat hazardous (6, 7). The large discrepancies observed in clinical correlations of AECAs may be ascribed to several variables that affect their measurement. For example, the choice of cells (with possible interdonor variability) or the use of cell membrane extracts, cell culture and fixation conditions, heating of serum samples (8), and the expression of results may affect the outcome.

While attempting to optimize cell fixation in such an ELISA, we identified another possible confounding factor: a substantial proportion of patient samples containing IgG antibodies against bovine serum proteins gave false-positive results in the AECAs test. Human antibodies reactive with animal proteins, including immunoglobul-
lins and common blocking agents such as cow’s milk, bovine serum albumin (BSA), and non-immune animal sera, are also designated as heterophile antibodies when they seemingly do not arise against a well-defined immunogen (9). These antibodies represent an often unrecognized source of interference in all immunoassays, potentially giving rise to false-positive results (10–14). The present study was therefore designed (a) to delineate heterophile antibody interference in our ELISA for AECAs, (b) to propose strategies for resolving the problem, and (c) to better characterize the antibodies involved. A heightened awareness of this type of interference on the part of investigators performing the AEC ELISA should improve its reliability, a prerequisite to better evaluate the role of AECAs in clinical practice and unravel their putative pathogenicity.

Materials and Methods

Patients and Controls

Serum samples, obtained from 60 patients with various non-organ-specific autoimmune disorders, were selected to cover a wide range of IgG AEC concentrations, from weakly to strongly positive. Of these, eight were part of a previous study, and their apoptosis-inducing effect on endothelial cells has been described (2). In addition, 30 patient sera that previously had been shown to react in ELISAs to bovine, but not human, β2-glycoprotein I (β2GPI) (15) were used to determine the prevalence of heterophile antibodies under study (see below) in this population. Sera from 45 healthy blood donors served as controls. All sera were stored at −20 °C until use.

Endothelial Cells

Fixed monolayers of the human endothelial hybrid cell line EA.hy 926 (a kind gift from C.S. Edgell, University of North Carolina, Chapel Hill, NC), obtained by fusing human umbilical vein endothelial cells with a human lung carcinoma, have been shown to be appropriate to detect AECAs by ELISA (3, 4). Cells were cultured at 37 °C and 5% CO2 in DMEM (Eurobio) supplemented with 100 mL/L heat-inactivated fetal calf serum (FCS; BioWhittaker), 2 mmol/L glutamine, and 50 mL/L hypoxanthine aminopterin thymidine (FCS medium). After cultures had reached confluency, cells were detached using a mixture of 1.25 g/L trypsin and 0.2 g/L EDTA (1:4, by volume) in Tris buffer (Eurobio) for 3 min at 37 °C, and then were washed once in FCS medium.

ELISAs for AECAs

EA.hy 926 cells were plated (1 × 104 cells/well in FCS medium) in flat-bottomed 96-well microtiter plates (Nunc). Confluent cell layers from 2- to 3-day-old cultures were washed with phosphate-buffered saline (PBS) and fixed with 100 μL of 1 g/L glutaraldehyde (or lower concentrations, as indicated) for 10 min at 4 °C. Alternatively, cells were fixed with 100 μL of absolute ethanol for 5 min at 4 °C. After three washings in PBS, plates were blocked with PBS containing 10 g/L BSA, and then were successively exposed to patient sera (100 μL of a 1:100 dilution in the same buffer) and to peroxidase-conjugated rabbit F(ab')2 anti-human IgG, IgM (both diluted 1:4000), or IgA (diluted 1:2000) antibodies (Dako), followed by incubation with o-phenylenediamine (0.2 g/L in 0.05 mol/L phosphate buffer, pH 5, containing 0.5 mL/L H2O2). Incubations were for 1 h at 37 °C, separated by three PBS washes. For each serum, the absorbance at 492 nm of control wells (blocked with PBS-FCS but without cells) was subtracted from the absorbance in the wells with EA.hy 926 cells to account for nonspecific binding. The mean + 3 SD of 45 control sera was taken as the threshold for positivity.

When we became aware of heterophile antibody interference in the above ELISA, we modified its format by diluting serum samples and conjugates in PBS containing 100 mL/L FCS and by blocking control wells with PBS-FCS.

Inhibition with Animal Sera

The ability of fluid-phase serum proteins from various animal species to inhibit the binding of patient heterophile antibodies to solid-phase-bound FCS proteins was measured by ELISA. In this FCS-ELISA—the interference assay—plates were coated with PBS containing 100 mL/L FCS for 4 h at 37 °C, and PBS containing 1 mL/L Tween 20 served as the blocking and wash buffer. Patient serum samples were diluted 1:100 in PBS-Tween alone or in PBS-Tween containing the indicated concentrations of competing animal serum. The development was conducted as described above in the AEC test.

Western Blot Analysis

FCS proteins (7 μL of crude FCS per lane) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% acrylamide gels under nonreducing conditions, followed by electrophoresis to nitrocellulose. Membranes were blocked for 2 h with PBS containing 1 mL/L Tween and were subjected to successive 1-h incubations with serum samples (1:100 in PBS-Tween) and the same anti-IgG conjugate as for ELISAs (1:4000 in PBS-Tween). Peroxidase activity was detected with diaminobenzidine.

Statistical Analysis

The Student t-test for paired data and the χ2 test were used when appropriate. P <0.05 was considered statistically significant.

Results

Cause of Spurious Increases in AEC Titer

In a study designed to optimize cell fixation in ELISA procedures for AEC detection, we noticed that apparent AEC activity did not decrease with cell loss in some patients’ samples (Fig. 1). This group, exhibiting spurious increases in antibody titers, included 12 of 60 (20%)
patient serum samples and was designated group I. Cell losses during the fixation step were estimated by phase-contrast microscopy, and reactivity with a monoclonal antibody (MAb) against a cell surface molecule of endothelial cells, thrombomodulin, that served as a probe for residual cell density. As shown in Fig. 1, the most important cell loss occurred in the absence of fixative and led to the strongest ELISA responses with group I sera. As a whole, all 12 group I sera showed increased relative absorbances that were negatively correlated with residual cell density, suggesting steric hindrance by cells and antibody recognition of noncellular targets. The remaining 48 patients (group II) possessed exclusively genuine AECAs, with a reactivity pattern resembling the one observed with the anti-thrombomodulin MAb, except for consistently increased binding after ethanol (vs glutaraldehyde) fixation.

To gain further insight into the understanding of the mechanism of the false positivity that characterized group I sera, we compared different treatments of the ELISA plate (Table 1). To avoid interference from added proteins, blocking and washing buffers contained Tween 20 alone in these experiments. Adherent EA.hy 926 cells were either detached with trypsin-EDTA or by exposure of their extracellular matrix (ECM) with detergent because antibodies to ECM components such as collagen and laminin have been reported in AECA-positive patients (1). The coating by EA.hy 926 cells in FCS-containing culture medium was sensitive to digestion with trypsin, which suggests the involvement of proteic structures in antigenicity. These proteins originated entirely from FCS and probably included BSA, as demonstrated when cells were omitted and replaced by crude FCS or purified BSA.

REDESIGN OF AECA-ELISA TO PREVENT HETEROPHILE ANTIBODY INTERFERENCE
We next investigated different strategies to solve the problem of interference by heteroantibodies against FCS proteins in our ELISA for AECAs. Typical results are shown in Fig. 2. We first washed EA.hy 926 cells twice and switched to serum-free DMEM before plating for ELISA. Although this approach was efficient for decreasing to baseline the binding of 9 of 12 group I sera when BSA served as sample diluent, it suffered from the requirement for approximately four times as many cells per well because the culture time was reduced to the few hours necessary to get firm cell adherence to the solid phase. We next evaluated the substitution of 100 mL/L FCS for the 10 g/L BSA used to dilute the test samples (Fig. 2B), based on the rationale that reactivity against bound FCS proteins should be preabsorbed. This clearly was the case, and spurious signals were totally eliminated in the nine group I sera that were negative when incubated with FCS-depleted washed cells. This observation was extended to a set of 30 patient samples possessing IgG specific for bovine (but not human) β2GPI, associated in 18 cases with IgG to FCS proteins (see below). Finally, the simple subtraction of the binding to FCS-coated wells (sample blank) from the binding to EA.hy 926-coated wells was inappropriate (at least in the absence of FCS in the sample dilution buffer) in view of the higher values of the former. Consequently, this practice would overcorrect specific antibody estimates in samples in which heteroantibodies coexist with genuine AECAs. Three such cases were identified in group I (group I subset), on the basis of reactivity profiles, which were identical to group II sera when FCS was used as sample diluent. Thus, the dilution of serum samples in PBS containing 100 mL/L FCS, along with the use of control wells coated with 100 mL/L FCS medium alone, were eventually chosen as the most convenient means of measuring AECAs without interference by heterophile antibodies.

PROPERTIES OF HETEROPHILE ANTIBODIES
Using a FCS-ELISA (the interference assay), designed to detect involved heteroantibodies, we sought to further characterize the heterophile antibodies in our samples. To assess the species specificity of IgG antibodies against FCS proteins, decreasing concentrations of a variety of competing animal sera were added to PBS-Tween at the antibody-binding step (Fig. 3). As expected, the heteroantibody interference was quantitatively inhibited by the addition of FCS or adult bovine serum, with 50% inhibi-
tion of binding reached at serum concentrations as low as 0.002–0.1%. These antibodies exhibited a broad specificity, recognizing serum proteins from goat, sheep, and to a lesser extent horse, although they bound less efficiently than their bovine counterparts. Only slight inhibition was achieved in most cases when concentrations of serum of human, pig, rabbit, and rat origin up to 50% were used.

IgG antibodies against bovine serum proteins, including BSA and $\beta_2$GPI, appear to be a common finding in healthy blood donors, the prevalence being 13–18% when direct ELISAs are used (Table 2). In addition, these types of antibodies often coexist in patient populations, and the frequencies observed in 30 patients selected for the presence of species-specific IgG to bovine $\beta_2$GPI, as well as in group I patients, largely exceeded the ones in controls. However, anti-BSA and anti-bovine $\beta_2$GPI antibodies contributed only marginally to the whole anti-FCS reactivity in double- or triple-positive sera, because fluid-phase BSA or $\beta_2$GPI caused poor inhibition in the FCS-ELISA, contrasting with total blockade in the homologous assays. Among the patients with anti-FCS antibodies shown in Table 2, the most prevalent isotype was IgG, alone in 14 cases, or associated with IgA, IgM, and IgA $+$ IgM in 12, 2, and 2 cases, respectively. Two patients possessed only IgM.

We also performed immunoblotting of FCS, using the 12 group I sera as the overlaying IgG antibodies. Seven of the sera were reactive to a limited number of proteic bands often shared by the sera, in particular at >250, 220, 160, 140 and 95 kDa. No attempt was made to identify the bands. There was no band detectable in the 66- and 50-kDa regions, which correspond to BSA and $\beta_2$GPI, respectively.

**Discussion**

The present study confirms that antibodies (mostly IgG and IgA) against animal serum proteins are widely distributed in human serum (10–12, 16), and it establishes their potential to give falsely increased AECA results, as detected by a cyto-ELISA. During unsuccessful attempts to set up an ELISA with unfixed endothelial cells, we incidentally noticed strong ELISA responses with some

![Fig. 2. IgG binding in various ELISA formats for AECAs, comparing sample dilution in PBS containing 10 g/L BSA (A) or PBS containing 100 ml/L FCS (B) and three types of coating. Wells were incubated for 6 h with medium containing 100 mL/L FCS (sample blank; ), or with EA.hy 926 cells (5 $\times$ 10^4 cells/well) resuspended, after being washed with PBS, in FCS-depleted culture medium ( ) or medium containing 100 mL/L FCS ( ). The plates were then washed twice in PBS and fixed with ethanol before probing with control and patient serum samples. A representative experiment is shown (mean $\pm$ SD of absorbance values) that included three sera in each category, i.e., group I (heteroantibodies only; I), group II (genuine AECAs only; II), and group I subset (genuine AECAs associated with heteroantibodies; I subset).](https://academic.oup.com/clinchem/article-abstract/46/2/273/5640512)
sera despite very low residual cell density, suggesting that the antibodies were not cell specific. This is reminiscent of the report by Westphal et al. (5) of sera that reacted strongly with the gelatin used to encourage endothelial cell adhesion, both in the healthy control and in the patient sera. These investigators noted that the signal in control wells sometimes overcame the test values in the presence of cells, in keeping with the behavior of samples from 12 of our patients (referred to as group I), in which IgG reacted to FCS from culture medium (Figs. 1 and 2). These observations stress the usefulness of running appropriate sample blank wells, the absorbance values of which are usually subtracted from those of the samples. However, the fact that the binding of group I sera was much higher in wells coated with FCS than in cell-seeded wells (Fig. 2A) casts doubt on the value of sample blank subtraction in these cases, with a risk of missing genuine AECAs that happen to coexist with heteroantibodies (e.g., group I subset). The approach that we selected for both circumventing the problem of false positives and properly detecting associated genuine AECAs is to include 100 mL/L FCS in the sample dilution buffer (Fig. 2B). This ability of FCS or adult bovine serum to inhibit, in a concentration-dependent manner, spurious signals from all assayed samples confirms that antibodies against bovine serum proteins were responsible for the interference. In this respect, it is intriguing that Craig et al. (17) observed blocking-specific background in immunoblot assays that was attributable to a reaction between endogenous IgG and the membrane blocking agent (i.e., milk or BSA), although sera were diluted in blocking buffer; reactivity against bound blocking proteins should have been absorbed. Interestingly, Mauracher et al. (18) had to use heat-denatured blocking proteins as the sample diluent to best reduce blocking-specific background in an ELISA. Alternatively, washing endothelial cells free of FCS before plating for ELISA also blocks heteroantibody interference, although it is more tedious than the above method. It thus is likely that none of the FCS proteins targeted by our series of heteroantibodies interacted with

**Table 2. Prevalence of IgG antibodies to crude FCS, BSA, and bovine b2GPI, as determined by direct ELISAs in control and patient groups.**

<table>
<thead>
<tr>
<th>Population (n)</th>
<th>100 mL/L FCS</th>
<th>5 g/L BSA</th>
<th>10 mg/L bovine b2GPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (12)</td>
<td>12 (100%)</td>
<td>8 (67%)</td>
<td>9 (75%)</td>
</tr>
<tr>
<td>Patients with anti-bovine b2GPI IgG (30)</td>
<td>18 (60%)</td>
<td>7 (23%)</td>
<td>30 (100%)</td>
</tr>
<tr>
<td>Healthy controls (45)</td>
<td>8 (18%)</td>
<td>6 (13%)</td>
<td>7 (15%)</td>
</tr>
</tbody>
</table>

*The threshold for positivity was set at 2 SD above the mean of 45 control sera in the three ELISAs.

*P <0.001 vs the corresponding frequencies in controls.

*Difference in frequency vs controls not significant.
cell membranes strongly enough to resist in vitro washes in PBS.

There has been recent emphasis on human anti-animal immunoglobulins, particularly human anti-mouse antibodies, as a cause of both positive and negative interferences in two-site immunoassays based on murine MAbs, in view of the increasing clinical application of the latter reagents for targeted imaging and immunotherapy. In most cases, so-called heterophile antibodies are weak, polyreactive antibodies produced in the absence of obvious exposure to animal proteins in a social or iatrogenic setting (9, 10). This raises the possibility that they arise from the natural process of antibody diversity (9). Of note is that heterophile antibodies, identified because of interference in interleukin-4 measurements by a two-site ELISA, have been reported to be under genetic control associated with self-tolerance and resistance to progression of type I diabetes (19). Alternatively, a major route by which animal protein antigens may be presented to the immune system and trigger antibody formation is the transfer of dietary antigens across the gut wall (10, 13). In keeping with this explanation is the increased incidence of antibodies to a variety of food antigens, including BSA and proteins from cow’s milk, which is higher in children than among older individuals or in association with pathological conditions such as celiac disease and selective IgA deficiency (16, 20). In our group I patients, favorable differential inhibition by FCS or adult bovine serum (Fig. 3) points to bovine proteins as the main immunogen, although clearly there was marked cross-reactivity with serum proteins from other animal species such as goat, sheep, and horse. Also in line with an antigen-driven response is the observation that antibodies to several bovine serum proteins frequently coexist in human sera (Table 2).

One puzzling fact from the literature dealing with AECAs is the variability of their prevalence in various diseases, and the basis for most of these discrepancies is presumably methodologic (1, 6, 7). We demonstrated here that endogenous antibodies to animal serum proteins frequently can interfere in any system that uses cultured cells as the solid-phase binder but that these antibodies are easily neutralized through assay redesign. This knowledge is of practical importance to achieve better interlaboratory agreement in the measurement of AECAs by ELISA.

Ronan Révélen is a recipient of a scholarship from the Communauté Urbaine de Brest, which is gratefully acknowledged. We thank C.S. Edgell, University of North Carolina, Chapel Hill, NC for providing us with the EA.hy 926 cell line.

References


