Detection of Antitrophoblast Antibodies in the Sera of Patients With Anticardiolipin Antibodies and Fetal Loss

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Women with anticardiolipin antibodies (ACLA) are at increased risk for fetal loss. One potential explanation for this outcome is that sera from these individuals contain antibodies reactive with trophoblast cells, which are involved in the establishment of the uteroplacental vasculature and maintenance of placental blood fluidity. To examine this hypothesis, we compared the incidence of trophoblast-reactive antibodies in 27 patients with ACLA and a history of fetal loss with that in 29 normal pregnant women. Sera from 20 patients, but only one control, contained trophoblast-reactive antibodies (P < .001). These antibodies were not directed against major histocompatibility class I antigens, and reacted with both term and first-trimester trophoblast cells. In most cases, sera from which ACLA were adsorbed by cardiolipin-containing liposomes maintained reactivity against cells. In addition, patient Ig fractions immunoprecipitated an ~62-kD protein from the trophoblast cell surface, stimulated the release of arachidonic acid and thromboxane A2 by trophoblasts, and inhibited the binding of prourokinase to trophoblast urokinase receptors. These observations show that sera from women with ACLA and a history of fetal loss contain antitrophoblast antibodies. These antibodies may be serologically distinct from ACLA, and may contribute to the pathogenesis of fetal demise.

The results of several studies show that women whose sera contains anticardiolipin antibodies (ACLA) are at increased risk for fetal loss. Many physicians regard the presence of ACLA in a patient with a previous spontaneous abortion as sufficient indication to initiate maternal therapy with corticosteroids, aspirin, or anticoagulants during subsequent pregnancies. However, many women with ACLA experience normal pregnancies, and prospective studies have not universally found ACLA to be independent predictors of fetal loss. In addition, discordance between the effect of maternal therapy on pregnancy outcome and the presence or titer of ACLA has been a finding common to numerous reports. These observations may indicate that only some ACLA have the potential to cause disease. However, no differences in the phospholipid specificities and/or affinities of ACLA in patients with fetal loss and those with normal obstetrical histories have been identified. Based on these findings, it is possible that factors other than, or in addition to, ACLA per se may contribute to the pathogenesis of fetal demise. Alternatively, only certain ACLA may be involved in the pathogenesis of this syndrome, although the specific characteristics of these antibodies have not yet been identified.

Examination of placentae from patients with ACLA-associated fetal loss provides insight into the pathogenesis of this disorder. These placentae may contain infarcts, and are commonly smaller than expected for gestational age. Histologic findings noted during a thorough microscopic analysis of involved placentae included decreased vasocoronal membranes and an increased incidence of villous bridging, fibrosis, and infarction. Similar findings have been reported in animal models of ACLA-associated fetal loss.

The delivery of blood to the developing fetus depends on the establishment of an adequate uteroplacental vasculature early in pregnancy, as well as the maintenance of blood fluidity within the placental intervillous spaces throughout gestation. Trophoblast cells are of central importance to the success of both of these processes. Therefore, the pathophysiology of ACLA-associated fetal loss may involve trophoblast damage or dysfunction, potentially mediated on an immune basis. Because sera from patients with ACLA may contain antibodies reactive with a number of cell types, including endothelial cells, platelets, and/or platelet-derived microparticles, we hypothesized that sera from some patients with ACLA-associated fetal loss might contain trophoblast-reactive antibodies, potentially capable of disrupting trophoblast-mediated activities critical to the success of pregnancy. To investigate this hypothesis, we compared the incidence of such antibodies in 27 women with ACLA and a history of fetal loss and 29 normal pregnant women.

MATERIALS AND METHODS

Materials: Antibodies used in enzyme-linked immunosorbent assays (ELISAs) and radioimmunoassays (RIAs) were from Organon Teknika-Cappel (Durham, NC); o-phenylenediamine (OPD) was from Zymed (San Francisco, CA); beef heart cardiolipin and A23187 were from Sigma (St Louis, MO) or Avanti Polar Lipids (Birmingham, AL); DNase type I, trypsin, glucose oxidase, and lactoperoxidase were from Calbiochem (La Jolla, CA); Percoll and chromatography columns were from Pharmacia-LKB Biotechnology (Piscataway, NJ); [125I] and [14C]-arachidonic acid was from New England Nuclear (Billericia, MA); tissue culture media, glutamine,
and antibodies were from Gibco-BRL (Grand Island, NY); fetal calf serum (FCS) was from Flow Laboratories (McLean, VA); anticytokeratin antibodies were from Boehringer-Mannheim (Indianapolis, IN) or Becton Dickinson (Mountain View, CA); anti-α human chorionic gonadotropin (hCG) antibodies were from ICN Biomedical (Lisle, IL); anti-β hCG, pregnancy-specific glycoprotein (SP-I), and vimentin antibodies were from Dako (Santa Barbara, CA); antiplacental alkaline phosphatase antibodies were kindly provided by Drs. M. Lafferty and H. Harris (University of Pennsylvania Department of Genetics, Philadelphia); monoclonal antibody (MoAb) 63D3 was purified from hybridoma conditioned medium. Purokinase was kindly supplied by Dr. Jack Henkin (Abbott Laboratories, Abbott Park, IL). RIA kits for thromboxane B2 (TXB2) and 6-keto-PGF1α were obtained from Amersham (Arlington Heights, IL).

Cell isolation and culture. Term trophoblasts were isolated and cultured as previously described. At least 97% of the cells isolated were trophoblasts, as judged by immunohistochemical staining using antibodies specific for cytokeratin 7 and 18, β-hCG, pregnancy-specific glycoprotein, and placental alkaline phosphatase, and by the absence of staining with antibodies specific for either vimentin or human monocytess. All studies were performed using primary cultures. Approximately 20 placentae were required to complete these studies, since each placenta yielded sufficient cells to measure trophoblast-reactive antibodies in five individual serum specimens.

First-trimester trophoblasts were isolated as previously described. These cells were characterized by their production of β-hCG, and by immunohistochemical staining with antibodies specific for placental alkaline phosphatase and cytokeratins 7, 8, and 18. Only cells from passages 1 through 4 were used in these studies.

Human umbilical vein endothelial cells (HUVEC) were isolated as previously described, and cultured in Medium 199 containing 10% FCS and 100 μg/mL crude endothelial cell growth factor.

Patients. The patient group included 27 patients with ACLA and histories of previous fetal loss (group A). Of these patients, 5 had experienced a single fetal loss (2 of which had occurred in the third trimester), 12 had experienced 2 consecutive prior fetal losses, and 10 had experienced 3 or more fetal losses. Overall, these 27 patients had experienced 66 episodes of fetal loss, of which 53 and 13, respectively, had occurred before or after 20 weeks of gestation. In 70 prior pregnancies, these patients had delivered only 4 live infants. Though a comprehensive evaluation for serologic evidence of systemic autoimmune disease was not performed, only 1 patient had clinical evidence of systemic lupus erythematosus, while another had a history of juvenile rheumatoid arthritis. All sera collected from these patients were obtained during pregnancy. The normal pregnant control group consisted of 29 pregnant women (group B) who had experienced a total of 43 successful pregnancies without fetal loss. The incidence of antitrophoblast antibodies in this group was not significantly different than that in a group of 15 women with normal obstetrical histories who were not pregnant at the time of blood donation (data not shown). An additional control group (group C) consisted of 12 pregnant patients with previously identified causes of fetal loss who did not have ACLA. This group included 8 patients with luteal-phase defects, 2 patients with uterine anomalies, 1 patient with chlamydial infection, and 1 patient with an incompetent cervix. A third control group consisted of 6 patients with ACLA detected after an initial episode of fetal loss who were observed prospectively during their next pregnancy (group D). During this pregnancy, each woman was treated in a nonrandomized fashion with either prednisone alone or prednisone combined with aspirin. Serum was collected from each woman during this subsequent pregnancy and antitrophoblast antibodies in these samples were correlated with pregnancy outcome. Three of the six women delivered viable infants.

Measurement of ACLA and lupus anticoagulants. IgG, IgA, and IgM ACLA were measured using a modification of a previously described RIA. Briefly, 96-well microtiter plates were coated with 25 μL of a solution containing 50 μg/mL of cardiolipin in ethanol. Ethanol was removed by overnight evaporation at 4°C, and wells were blocked with phosphate-buffered saline (PBS) containing 1% gelatin. The plates were then incubated sequentially with a 1:1 dilution of patient or normal serum, goat antihuman IgG, IgA, or IgM, and 125I-labeled staphylococcal protein A. After washing, the radioactivity remaining in each well was determined. Patient serum samples were tested to contain ACLA if they deposited at least 2 SDs more IgG, IgA, or IgM onto cardiolipin-coated wells than the mean amounts of these IgGs deposited by 56 normal control sera. ACLA were measured in 13 patients on at least two occasions separated by 6 or more weeks. Although ACLA levels showed some variation between measurements in 10 of these patients, all patients remained positive in the ACLA assay on both occasions. For the purposes of this study, the ACLA value corresponding to the time at which serum was obtained for measurement of antitrophoblast antibodies is reported. Sera from 10 additional patients were also analyzed simultaneously using the anticardiolipin ELISA, as described by Loizou et al, and the anticardiolipin RIA described above. The cutoff for positivity in the RIA (2 SD) was found to correspond to a value ≥25 GPL and/or 10 MPL units of ACLA activity in the ELISA, calibrated using the Rayne Institute standards (obtained from Dr. E. Nigel Harris, University of Louisville, Louisville, KY). Furthermore, sera from each of 10 of the initial patients found to contain ACLA using the RIA produced moderately to strongly positive results in the ELISA as well. ELISAs were also used to determine the levels of ACLA in sera before and after adsorption with cardiolipin-containing liposomes.

Patient plasma was considered to contain a lupus anticoagulant if the activated partial thromboplastin time (aPTT) was prolonged by ≥5 seconds above the normal range and did not correct after addition of an equal volume of normal plasma, the tissue thromboplastin inhibition ratio was ≥1.3, and the prolonged aPTT was completely corrected by the addition of freeze-thawed platelets. Measurement of cell-reactive antibodies. Antibodies against cultured trophoblasts and endothelial cells were also measured by ELISA. Briefly, cells were cultured in 96-well microtiter plates for 24 hours after isolation (or, in the case of first-trimester trophoblasts, for 24 hours after their last passage). They were then washed with Hank's balanced salt solution containing 1% bovine serum albumin (HBSS-BSA), and nonreactive sites were blocked by a 30-minute incubation in HBSS containing 3% BSA and 10% FCS. Cells were then incubated for 90 minutes with a 1:5 dilution of patient sera, washed with HBSS-BSA, and incubated with peroxidase-conjugated goat antihuman IgG, IgA, or IgM. After washing, bound antibody was measured by adding OPD (1 mg/mL), and measuring A492 in individual wells using a microplate reader (Molecular Devices, Menlo Park, CA). All points were determined in triplicate, and all serum samples were assayed on at least two separate occasions. Samples that deposited at least 2 SD more IgG, IgA, or IgM on trophoblasts than the mean amount of these IgGs deposited by serum from 29 normal pregnant women were considered to contain antitrophoblast antibodies.

Preparation of Ig fractions and Fab (Fab′) fragments. Several studies were performed to further characterize the reactivity of patient IgG with trophoblasts. First, we determined whether the trophoblast-reactive IgG in patient serum was in the form of monomeric IgG or contained within immune complexes. Serum from two patients and two controls was separated by gel filtration chromatogra-
by centrifugation, resuspended in 0.5 mL of distilled water, dia-
sorbance at 450 nm caused by the serum sample before and after
trophoblast function in vitro, Ig fractions were prepared from pa-
serum samples were incubated sequentially with 3 aliquots (-0.3
lyzed extensively against PBS, and adjusted to a final volume
calculated as follows:
trophoblast-reactive antibodies remaining in the samples after these
(Ig2) to trophoblasts was compared using an ELISA.

Finally, to determine the effects of antitrophoblast antibodies on
trophoblast function in vitro, Ig fractions were prepared from pa-
tient serum and pooled serum from five normal pregnant patients.
Ig fractions were prepared by precipitation of serum with ammo-
nium sulfate (40% final concentration). Precipitates were collected
by centrifugation, resuspended in 0.5 mL of distilled water, dia-
lized extensively against PBS, and adjusted to a final volume of
2 mL. These fractions were used at a dilution of 1:20 in studies inves-
tigating their effects on trophoblast function.

Liposome adsorption studies. To determine whether removal of
ACLA from patient sera affects their reactivity with trophoblasts,
sample sera were incubated sequentially with 3 aliquots (~0.3
mL per incubation) of cardiolipin-containing liposomes, prepared
previously described.39 The relative amounts of cardiolipin and
trophoblast-reactive antibodies remaining in the samples after these
incubations were then measured. Specific removal of ELISA reactiv-
ity attributable to ACLA or trophoblast-reactive antibodies was cal-
culated as follows: % removal = (A450PRE - A450POST/A450PRE
- A450CTRL) x 100, where A450PRE and A450POST refer to the ab-
sorbance at 450 nm caused by the serum sample before and after
liposome adsorption, respectively, and A450CTRL = mean absorb-
bance at 450 nm of five control sera obtained from normal preg-
nant women, which were included with each ELISA assay.

Lymphocyte cytotoxicity assay. Sera from four patients with
ACLA were screened for the presence of HLA-reactive antibodies
by Dr Jay Herman (Temple University School of Medicine, Phila-
delphia, PA), using a lymphocytotoxicity assay.37 Briefly, lympho-
cytes of known major histocompatibility complex (MHC) type
were incubated in round-bottom 96-well microtiter plates with pa-
tient or control sera and rabbit complement. Trypan blue was then
added, the cells washed, and the percentage of nonviable cells det-
ected by dye uptake. Sera that lysed greater than 60% of the
target cells were considered to contain HLA-reactive antibodies.

Immunoprecipitation studies. To determine whether tropho-
blast-reactive antibodies recognize proteins expressed on the cell
surface, term trophoblasts were isolated and cultured in serum-free
medium for 36 hours before labeling of cell-surface proteins with
25I.34 Trophoblast cell extracts were then prepared in a buffer con-
taining 1% Triton-X-100, and immunoprecipitated with control or
patient Ig fractions as previously described.33 Equal amounts (in
disintegrations per minute) of immunoprecipitated, radiolabeled
cell-surface proteins, added to parallel lanes, were analyzed using
10% SDS-PAGE under nonreducing conditions,59 and detected using
autoradiography.

Effect of antitrophoblast antibodies on calcium ionophore-me-
diated arachidionate release. These studies were performed as pre-
viously described by Scherer et al.60 Cultured trophoblasts were
labeled with 3H-arachidonic acid (0.5 μCi/mL) for 4 hours at 37°C.
Cells were then washed and incubated for 50 minutes with either
fresh serum-free medium or medium containing 20-fold dilutions
of Ig fractions prepared from patient serum or pooled serum from
normal pregnant women. Cells were then washed with warm PBS
and incubated for an additional 30 minutes in serum-free medium
containing 5 μmol/L A23187. Measurement of 3H-arachidonic acid
released into conditioned medium was determined by scintilla-
tion counting. Spontaneous release of radioactivity in the absence
of A23187 was less than 1.4% of the total amount of radioactivity
incorporated into cells.

Effect of antitrophoblast antibodies on the secretion of thrombo-
axane A2 (TXA2) and prostacyclin (PGI2). In these studies, tropho-
blasts were cultured for 24 hours in serum-free medium contain-
ing 50 μmol/L arachidonic acid, in the absence or presence of 20-fold
dilutions of Ig fractions prepared from either patient serum or
pooled serum from normal pregnant women. Medium was then
collected, centrifuged at 2,500 g for 10 minutes, and frozen at
-80°C until analyzed. Concentrations of TXA2 and PGI2 were
determined by measuring their stable metabolites, TXB2 and 6-keto-
PGF1α, using commercial RIAs.

Effect of Ig fractions on binding of 125I-prourokinase to tropho-
blasts. To measure the effect of patient and control Ig on the bind-
ing of 125I-prourokinase to trophoblasts, cells were cultured in 96-
well microtiter plates for 24 hours after isolation, then briefly
incubated in 50 mmol/L glycine-HCl, 0.1 mol/L NaCl, pH 3.0, to
evoke endogenous prourokinase.61 Trophoblasts were then washed
and incubated for 1 hour at 37°C with a 20-fold dilution of Ig
fractions prepared from either patient serum or pooled serum from
normal pregnant women. Cells were then chilled to 4°C, washed,
and incubated for 3 hours in PBS containing 0.1% BSA and 2.5
mmol/L 125I-prourokinase. At the end of this incubation period,
cells were washed and cell-bound radioactivity determined.62
Specific binding of 125I-prourokinase to the cells in the absence
of Ig was measured simultaneously in the presence of 250 mmol/L unlabeled
prourokinase.63

Statistics. The incidence of trophoblast-reactive antibodies in
control and patient sera was compared using a Student’s t-test. Con-
idence intervals were calculated as described by Simon.53 Correla-
tion coefficients and their significance were determined using the
Primer of Biostatistics software program (McGraw-Hill Book

RESULTS

Incidence of cardiolipin and trophoblast-reactive antibod-
ies in patient sera. Sera from all patients contained either
IgG, IgA, or IgM ACLA. The most common pattern of
ACLA isotypes observed was the simultaneous presence of
IgG and IgM ACLA, which was present in 17 patients. Iso-
lated IgG ACLA were present in five sera. These figures
contrast with a 2% incidence of ACLA in healthy pregnant
women studied concurrently, using the same ACLA assay.64
Sera from only four patients contained lupus anticoag-
ulants.

We next determined the incidence of antibodies in pa-
tient and control serum that reacted with trophoblasts iso-
lated from term placentae (Fig 1). IgG, IgA, or IgM antitro-
hoblast antibodies were detected in 10, 9, and 9 patient
sera, respectively, versus 0, 0, and 1 sera from normal preg-
nant women (P = .001, .002, and .010 for IgG, IgA, and
IgM, respectively). The isotype of antitrophoblast antibod-
ACLA AND ANTITROPHOBLAST ANTIBODIES

Fig 1. Deposition of Ig on term trophoblasts by patient and control sera. Trophoblast-reactive IgG, IgA, and IgM in patient and control sera was measured by ELISA, as described. In this figure, the ordinate depicts the amount of Ig deposited on cells (in SDI), and the abscissa depicts the analysis of Ig binding by specific isotype. The dotted horizontal lines denote \( \pm 2 \) SD from the mean amount of Ig deposited on trophoblasts by sera from normal pregnant women.

ies often differed from that of ACLA present in the same serum. For example, of 4 patients with isolated IgG ACLA, 2 had IgG, 1 had IgA, and 1 had IgM antitrophoblast antibodies (Table 1). Overall, 20 of 27 patient sera (74%; 95% confidence interval [CI] = 57% to 86%) versus 1 of 29 sera from healthy pregnant controls (3.4%; 95% CI 3.0% to 9.4%) contained antibodies of one or more isotypes reactive with term trophoblasts \( (P < .001) \).

To further assess the significance of these observations, two additional patient groups were studied. First, we determined the incidence of antitrophoblast antibodies in women who had experienced prior fetal losses for which a cause other than ACLA had been identified (group C). The amount of antitrophoblast antibody in these sera was compared with that in pooled serum from five normal pregnant women, as well as with that in serum from a patient known to have antitrophoblast antibodies, used as a positive control. Serum from the patients in group C deposited quantities of Ig on trophoblasts which were similar to that deposited by the pooled normal serum, and markedly less than that of a patient known to contain antitrophoblast antibodies (Table 2). These results suggest that antitrophoblast antibodies occur preferentially in a select group of patients with ACLA and unexplained fetal loss, and do not simply reflect a maternal immune response to prior unsuccessful pregnancies.

Next, the relationship between antitrophoblast antibodies and pregnancy outcome was studied prospectively in a small number of patients. The identification of a large cohort of women with ACLA before their first pregnancy was not practical because of the infrequent occurrence and low titer of such antibodies in the normal population. Therefore, we instead collected serum from six patients with ACLA identified as a consequence of prior fetal loss, who again became pregnant during the course of this study (group D). Each patient was treated with either prednisone alone, or prednisone combined with aspirin, in a nonrandomized manner. The level of antitrophoblast antibodies in these patients was measured during pregnancy and compared with fetal outcome. The amount of IgG antitrophoblast antibody in the three patients whose pregnancy resulted in fetal loss was greater than that in each of the sera from the three patients who experienced successful pregnancies (Table 3). The levels of IgA antitrophoblast antibodies were also higher in 2 of the 3 patients with adverse outcomes, though no differences in the levels of IgM antitrophoblast antibodies were observed between the two patient groups. Although the statistical significance of this data cannot be determined because of the small number of patients studied, these results nevertheless support the hypothesis that elevated levels of antitrophoblast antibodies may contribute to a poor pregnancy outcome.

Finally, because many of the pathophysiologic events involved in ACLA-associated fetal loss may occur in the first trimester, we determined whether 12 patient sera that contained antibodies reactive with term trophoblasts displayed similar reactivity with trophoblasts isolated from first-trimester placentae. Overall, 10 of these sera (83%; 95% CI = 59% to 91%) displayed reactivity against first-trimester trophoblasts, whereas none of 12 control sera, obtained from normal pregnant women, contained such antibodies (data not shown).

Characterization of trophoblast-reactive IgG. Because trophoblasts express receptors for the Fcγ portion of IgG, which may bind IgG-containing immune complexes, we determined whether the observed binding of patient IgG to trophoblasts was attributable to the activity of specific, target-binding They have previously been described

Table 1. Isotypes of ACLA and Antitrophoblast Antibodies in Group A Patients in Whom Both Types of Antibodies Were Present \( (n = 20) \)

<table>
<thead>
<tr>
<th>ACLA Isotype</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
<th>IgG, IgA</th>
<th>IgG, IgM</th>
<th>IgA, IgM</th>
<th>IgG, IgA, IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgA</td>
<td>0</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgG, IgA</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG, IgM</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>IgA, IgM</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IgG, IgA, IgM</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
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</table>
monomeric antibody, or to the presence of immune complexes in patient serum. Serum from two patients and two normal pregnant controls were separated by gel filtration, and the amount of trophoblast-reactive IgG in each fraction was measured. Figure 2 depicts the relative amounts of trophoblast-reactive IgG in both patient sera was found in fractions containing proteins with estimated molecular weights of 150 to 160 kD, consistent with that of monomeric IgG. However, high molecular weight fractions (>750 kD) from both patient, but neither control sera, also contained trophoblast-reactive IgG. Gel filtration fractions from both patients were also tested for reactivity with human umbilical vein endothelial cells, which do not express Fcy receptors. Endothelial cell reactivity was found only within fractions containing proteins with estimated molecular weights of 150 to 160 kD (data not shown). These findings suggest that the reactivity of patient IgG with trophoblasts is due primarily to specific F(ab')2-mediated interactions of monomeric IgG with trophoblast cell-surface antigens.

This conclusion is further supported by the results of experiments in which the binding of F(ab')2 fragments of IgG from three patients with antitrophoblast antibodies and those derived from the pooled serum of five normal pregnant women were compared. Approximately 2.9-, 2.2-, and 1.5-fold more patient-derived F(ab')2 fragments bound to trophoblasts than F(ab')2 fragments prepared from the normal pool (data not shown). These results are consistent with the presence of specific trophoblast-reactive antibodies in patient serum.

**Antigenic specificity of trophoblast-reactive antibodies.** We next performed several studies to determine the nature of the antigenic sites on the cell surface recognized by trophoblast-reactive antibodies. First, we first determined whether these antibodies bound exclusively to trophoblasts or reacted with other components of the uteroplacental vasculature, such as endothelial cells. The reactivity of serum from 7 patients (5 with antitrophoblast antibodies and 2 without) and 10 normal pregnant women (used to determine a normal range for each cell type in this set of experiments) with term trophoblasts and HUVEC was determined in parallel. A strong correlation between the amounts of IgG, IgA, and IgM deposited on trophoblasts and HUVEC by these sera was observed (r = .936, .788, and .956, respectively). However, in the case of IgG, the relative amount of antibody deposited on trophoblasts (expressed in standard deviations from the mean amount of IgG deposited by the control sera) was greater than the amount deposited on endothelial cells (Fig 3).

**Table 2. Deposition of IgG, IgA, and IgM on Trophoblasts by Serum From Patients With Fetal Loss of Known Etiology, and No ACLA**

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
<th>$A_{492}$ Ratio (patient serum/normal pool)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6</td>
<td>1.9</td>
<td>1.0</td>
<td></td>
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<tr>
<td>2</td>
<td>1.0</td>
<td>2.1</td>
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<tr>
<td>3</td>
<td>1.2</td>
<td>1.3</td>
<td>1.1</td>
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<tr>
<td>4</td>
<td>1.2</td>
<td>1.6</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.2</td>
<td>3.1</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.1</td>
<td>4.1</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.6</td>
<td>1.8</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.8</td>
<td>1.3</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.7</td>
<td>2.2</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.8</td>
<td>0.8</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.8</td>
<td>1.9</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.0</td>
<td>0.9</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Average (patient)</td>
<td>1.0</td>
<td>1.9</td>
<td>1.0</td>
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</tr>
<tr>
<td>Positive control*</td>
<td>4.4</td>
<td>11.8</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

* This corresponds to 4.1, 3.9, and 3.0 SDs more IgG, IgA, and IgM, respectively, on trophoblast cells than the mean amount of these Igs deposited by sera from 29 normal pregnant women.

**Table 3. Deposition of IgG, IgA, and IgM on Trophoblasts by Serum From Six Patients With ACLA Studied Prospectively**

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
<th>$A_{492}$ Ratio (patient serum/normal pool)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>3.6</td>
<td>1.3</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>9.6</td>
<td>10.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td>3.4</td>
<td>5.3</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>2.1</td>
<td>1.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>0.8</td>
<td>1.2</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td>1.6</td>
<td>0.8</td>
<td>0.9</td>
<td></td>
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<tr>
<td>Positive control*</td>
<td>7.2</td>
<td>4.0</td>
<td>2.1</td>
<td></td>
</tr>
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* This corresponds to 4.1, 3.9, and 3.0 SDs more IgG, IgA, and IgM, respectively, on trophoblast cells than the mean amount of these Igs deposited by sera from 29 normal pregnant women.
ACLA AND ANTITROPHOBLAST ANTIBODIES

Though normal human trophoblasts express only the monomorphic MHC determinant, HLA G,70-72 we examined the possibility that patient sera may contain alloantibodies that recognize MHC antigens either bound to trophoblast cells or potentially expressed by these cells under pathologic conditions.73 Four patient sera containing antitrophoblast antibodies were analyzed for the presence of such MHC-reactive alloantibodies. Of these, two displayed no MHC reactivity, a third displayed only weak reactivity against HLA B-18 (but reacted strongly with all 16 trophoblast cultures against which it was tested), and a fourth displayed broad HLA reactivity. These results show that MHC alloreactivity cannot account for the trophoblast reactivity in these sera.

Lyden et al74 have demonstrated staining of trophoblasts within histologic sections by monoclonal antiphospholipid antibodies. To determine whether the trophoblast reactivity in the sera of patients we studied was attributable solely to ACLA, we performed several experiments. First, we analyzed the statistical relationship between the relative amounts of trophoblast and cardiolipin-reactive antibodies (measured in standard deviations) in individual sera. Weak correlations between these two parameters were noted for all three antibody isotypes (r = .28, .42, and .59 for trophoblast and cardiolipin-reactive IgG, IgA, and IgM, respectively, when only patient sera that contained antitrophoblast antibodies were analyzed). Only the correlation between IgM antitrophoblast and ACLA was statistically significant (P = .42, .07, and .005 for IgG, IgA, and IgM, respectively). These findings, taken together with the observations that the isotypes of ACLA and antitrophoblast antibodies in individual sera may differ (Table 1), and that IgG ACLA were present in 7 of 7 sera that lacked trophoblast-reactive antibodies, suggest that ACLA and antitrophoblast antibodies are often not identical.

To examine this issue further, we next used cardiolipin-containing liposomes to adsorb ACLA from three patient sera. The residual cardiolipin and trophoblast reactivity was then determined in the adsorbed serum (Fig 4). Adsorption of serum from one patient with both IgG and IgM ACLA and antitrophoblast antibodies removed ~100% of the IgG and ~60% of the IgM ACLA, while reducing levels of cell-reactive IgG by less than 6% and not affecting cell-reactive IgM (Fig 4A). In a second patient with both IgG and IgM ACLA and antitrophoblast antibodies, adsorption of sera with liposomes removed 100% of the IgG and 69% of the IgM ACLA. Similar to our observations with the first patient, the postadsorption levels of IgG antitrophoblast antibodies in this sera were reduced by only 7.5%; however, the levels of IgM antitrophoblast antibodies were reduced by 89% (Fig 4B). In a third patient with only IgG ACLA and antitrophoblast antibodies, complete removal of ACLA from the sera did not affect antitrophoblast antibody levels (Fig 4C). These results also suggest that antibodies other than ACLA per se may contribute to trophoblast reactivity.

Finally, we determined whether trophoblast-reactive antibodies recognized proteins expressed on the trophoblast cell surface. After labeling of trophoblast cell-surface proteins with 125I, trophoblast extracts were prepared, and labeled proteins immunoprecipitated with Ig fractions prepared from three patients and two normal pregnant women. Both patient and control Ig fractions nonspecifically precipitated several proteins of approximately 85 to 105 kD (not shown). However, all three Ig fractions prepared from patient sera also precipitated an ~62-kD protein that was not precipitated (or precipitated only in barely detectable amounts) by control Ig fractions (Fig 5).

Effects of patient Ig on trophoblast function in vitro. To determine potential mechanisms by which antitrophoblast antibodies might contribute to the pathogenesis of placental insufficiency and fetal loss, we examined their effects on the production of agents potentially important in the regulation of placental blood flow and maintenance of placental blood fluidity. First, we determined whether patient Ig fractions affected trophoblast prostaglandin metabolism by measuring their effect on calcium ionophore-mediated arachidonic acid release and the production of TXA2 and PGI2 by trophoblasts. Second, we measured the effect of these fractions on the binding of urokinase to trophoblast urokinase receptors, a process that appears necessary for plasmin-dependent remodeling of the maternal uterine vasculature, as well as the maintenance of blood fluidity within the placental intervillous spaces by trophoblasts.

In contrast to previous studies examining the effects of ACLA on HUVEC,60 we observed that 3 of the 5 patient Ig fractions studied stimulated (28%, 35%, and 64%, respectively), rather than inhibited, A23187-induced arachidonic acid release from trophoblasts. Arachidonic acid release was
Fig 4. Effect of liposome adsorption on levels of trophoblast-reactive antibodies in patient sera. Patient sera was adsorbed with cardiolipin-containing liposomes, as described in Materials and Methods. The residual reactivity of adsorbed sera with cardiolipin (A) and trophoblasts (B) was then determined. (A) through (C) depict the results of experiments using sera from three different patients. Patients (A) and (B) had both IgG and IgM antitrophoblast and ACA. Patient (C) had only IgG antitrophoblast and ACA. Results depict the mean values of two separate experiments. ATA, antitrophoblast antibodies.

not affected by the other two patient Ig fractions, or that derived from pooled normal pregnant serum. Similarly, 4 of the 5 patient Ig fractions stimulated the secretion of TXA₂ by trophoblasts (18%, 23%, 23%, and 45%, respectively), while Ig fractions prepared from the fifth patient as well as from serum pooled from normal pregnant women had no effect. All three Ig fractions that stimulated ³H-arachidonic acid release also caused increased TXA₂ production. Furthermore, the increase in TXA₂ production was not compensated by a concordant increase in the production of prostacyclin. Indeed, the secretion of prostacyclin by trophoblasts was inhibited by 1 of the 5 patient Ig fractions, while it was unaffected by the others. Thus, the net effect of at least 3 of the 5 patient Ig fractions was to cause an increase in the production of TXA₂ relative to prostacyclin by trophoblasts.

In the second set of experiments, we examined the effects of patient Ig on the binding of ¹²⁵I-prourokinase to trophoblasts. Though the Ig fraction derived from the pooled sera of normal pregnant women inhibited ¹²⁵I-prourokinase binding to a limited extent (12%), binding was inhibited to a much greater extent by each of the five patient-derived Ig fractions (Fig 6). Two of these fractions reduced the binding of ¹²⁵I-prourokinase to levels similar to that observed in the presence of a 100-fold molar excess of unlabeled prourokinase. These results were confirmed when identical concentrations of IgG purified from two patients and pooled normal pregnant serum were compared (data not shown).

**DISCUSSION**

The results of this study demonstrate that sera from many patients with ACLA and a history of fetal loss contain antitrophoblast antibodies. These antibodies do not occur with increased frequency in pregnant women without ACLA who have experienced normal pregnancies, nor in those with histories of fetal loss accounted for by known hormonal or anatomic abnormalities. Furthermore, in the limited number of patients with ACLA studied prospectively (n = 6), the levels of IgG antitrophoblast antibodies were lower in the three patients who experienced subsequent successful pregnancies compared with the three who did not. Taken together, these results suggest that antitrophoblast antibodies occur only in a select group of women with fetal loss, which is unexplained other than by the presence of ACLA.

The broad reactivity of patient sera with term trophoblasts isolated from multiple placentae and the complete absence of cytotoxic anti-MHC antibodies in 2 of the 4 patient sera tested (with only weak reactivity against a single MHC epitope in a third) suggests that neither recognition of MHC nor polymorphic antigens such as TLX/C8 binding protein²⁵,²⁶ by the trophoblast-reactive antibodies accounted for our results. In the case of IgG, most of the trophoblast reactivity was mediated through the F(ab')₂ domain of monomeric antibody, although binding of high molecular weight IgG-containing immune complexes to trophoblast Fcγ receptors contributed to a limited extent. The results of liposome adsorption studies show that trophoblast-reactive antibodies may be serologically distinct from ACLA, although it appears that some ACLA, particularly those of the IgM isotype, may bind directly to trophoblasts.²⁴ These observations are consistent with previous studies from our laboratory²³ and others²⁰-²² concerning the antigenic specificity of endothelial cell-reactive antibodies in patients with ACLA, and are supported by the results of our immunoprecipitation experiments, which suggest that
Fig 5. Immunoprecipitation of $^{125}$I-labeled trophoblast cell-surface proteins by patient and control Ig fractions. Term trophoblast cell-surface proteins were labeled with $^{125}$I, and cell extracts prepared in a buffer containing 1% Triton-X-100. Aliquots of trophoblast extract were immunoprecipitated with control (lanes 1 and 2) or patient Ig fractions (lanes 3, 4, and 5), followed by rabbit antihuman Ig preincubated with protein-A Sepharose. Precipitates were solubilized in SDS sample buffer and analyzed using 10% SDS-PAGE (under nonreducing concentrations) and autoradiography. Migration of molecular weight standards in kilodaltons is shown on the left.

trophoblast-reactive antibodies recognize specific cell surface (glyco)proteins. The correlations between the reactivity of these antibodies with trophoblasts and endothelial cells, as well as the results of our previous studies in which an ~70-kD protein was immunoprecipitated from the endothelial cell surface by Ig fractions from patients with antiphospholipid antibodies and a history of thrombosis, suggest that similar proteins expressed by both cell types may be recognized by cell-reactive antibodies in patients with ACLA.

In contrast to a previous report, we did not detect trophoblast-reactive antibodies in sera from normal pregnant women; this discrepancy may be due to our study of intact, viable trophoblasts rather than glutaraldehyde-fixed syncytiotrophoblast plasma membrane vesicles. However, our studies are consistent with the findings of Grimmer et al, who detected trophoblast-associated IgM on examination of placental sections from a patient with a lupus-like disorder who experienced an intrauterine fetal death; deposition of IgM on the trophoblast cell layer of normal placentae by sera from this patient was also documented by indirect immunofluorescence. In addition, our studies extend those of Hasegawa et al, who detected cytotoxic antitrophoblast antibodies in 9 of 15 patients whose prior pregnancies were complicated by intrauterine growth retardation, of whom also had ACLA.

Our findings also extend those of Lyden et al, who detected binding of monoclonal IgM antiphospholipid antibodies to trophoblast within histologic sections. Reactivity against trophoblast correlated with the antiphosphatidylserine (PS) activity of the antibodies. Based on that data, these investigators have proposed that exposure of PS on the trophoblast surface during the processes of cell fusion and differentiation provides the epitope recognized by antiphospholipid antibodies. However, whether the monoclonal antiphospholipid antibodies used in these studies cross-react with nonphospholipid molecules such as prothrombin, glycoprotein I, DNA, or cytoskeletal proteins was not reported. Furthermore, the relationship between these IgM MoAbs and naturally occurring human antiphospholipid antibodies is uncertain. Our findings, though they support the observation that some ACLA may bind directly to trophoblast cells, suggest that in other cases trophoblast-reactive antibodies may be serologically distinct from those antibodies with primary specificity for negatively charged phospholipid. Phospholipid and trophoblast-reactive antibodies may belong to a spectrum of antibodies with varying affinity for phospholipid, phospholipid and protein-containing epitopes. The antibodies with highest avidity for cells may recognize primarily (glyco)protein epitopes and lie at one end of this spectrum, while those with greatest affinity for phospholipid, lying at the other end, may include the antibodies usually detected in clinical assays using immobilized cardiolipin. In between these extremes may lie antibodies that cross-react with both phospholipid and nonphospholipid.
epitopes. It is possible that the distribution of these antibodies in the sera of individual patients differs. However, this hypothesis can only be addressed by more sophisticated biochemical and molecular characterization of phospholipid and cell-reactive antibodies, as well as the cellular epitopes with which the latter react.

Previous studies have shown that purified IgG from patients with a history of ACAI-associated fetal loss causes fetal resorption when injected into pregnant mice. Although the ACAI IgG within these preparations was assumed to be of primary importance in the pathogenesis of resorption, it is uncertain whether other pathogenic antibodies with distinct reactivity against trophoblast or endothelium were present simultaneously. The potential importance of antiphospholipid antibodies per se in the induction of fetal loss is suggested by the observation that resorption was also induced by an MoAb reactive with cardioplin and DNA. However, the pathophysiologic events responsible for fetal resorption in this murine model have not been defined, and the cellular epitopes with which the monoclonal antiphospholipid antibodies may react have not been investigated. Additional studies will be required to clarify the pathophysiology, and establish the relevance, of this interesting model of fetal demise to ACAI-associated fetal loss in humans.

The role of phospholipid-reactive antibodies per se in the pathogenesis of ACAI-associated fetal loss in humans must also be reconsidered in the context of several recent observations. For example, the fact that many women with ACAI experience entirely normal pregnancies remains unexplained. Furthermore, the specific characteristics of ACAI in individual patients, such as titer, avidity, or phospholipid specificity have been shown to be of no use to date in predicting the success of pregnancy in individual patients. These observations suggest that factors other than, or in addition to, ACAI may be important in the pathogenesis of fetal loss in some individuals. Our results suggest that one such factor may be antitrophoblast antibodies. However, large, prospective clinical studies will be required to more clearly define the clinical implications of these findings.

However, our studies do suggest several potential mechanisms by which trophoblast-reactive antibodies might contribute to the pathogenesis of fetal demise. First, we found that Ig fractions from 3 of the 5 patients studied stimulated trophoblast phospholipase A2 activity, as measured by the release of 3H-arachidonic acid in response to the calcium ionophore A23187. This result differs from the previously reported inhibitory effect of antiphospholipid antibodies on endothelial cell phospholipase A2, possibly because of the presence of additional cell-reactive antibodies in the patients that we studied, or to cell-specific differences in the sensitivity of phospholipase A2 to cell- or phospholipid-reactive antibodies. Ig fractions from these same individuals, as well as an additional patient, also stimulated the production of TXA2 by trophoblasts; this observation, taken together with the failure of these fractions to cause an accompanying increase in trophoblast prostacyclin production, suggests that the arachidonic acid released caused by phospholipase A2 stimulation is preferentially used by trophoblasts as a substrate for TXA2 synthesis. In vivo, body-induced stimulation of the production of TXA2, a vasoconstrictor and platelet agonist, without accompanying increases in the production of prostacyclin, a vasodilator and inhibitor of platelet activation, may lead to vasoconstriction of the uterine spiral arteries with resultant placental ischemia. Similarly, an increased ratio of TXA2 to prostacyclin in the placental intervillous spaces may promote platelet adhesion and activation, leading to placental thrombosis. Of course, the effects of antitrophoblast antibodies on trophoblast prostaglandin metabolism in vivo may not correlate directly with their activity in vitro. However, similar effects of IgG fractions from patients with ACAI-associated fetal loss on the production of prostacyclin and TXA2 by placental explants, in which trophoblasts maintain their physiologic contacts with neighboring cells and extracellular matrix, have also been reported.

Our observation that Ig fractions from patients with antitrophoblast antibodies inhibit the binding of pronokinin to trophoblast urokinase receptors suggests a second mechanism by which these antibodies might contribute to the pathogenesis of fetal loss. Expression of uPA activity on the surface of invading extravillous trophoblast early in pregnancy may facilitate the remodeling of the uterine vasculature by these cells, a process essential to the establishment of a vascular system adequate to support the needs of the developing fetus. Furthermore, the maintenance of placental blood fluidity and prevention of fibrin accretion within the placental intervillous spaces throughout gestation may depend on the expression of uPA activity by villous trophoblast, which line the placental intervillous spaces. Disruption of either of these processes by antitrophoblast antibodies may lead to a situation in which placental blood flow and nutrient delivery is insufficient to meet the metabolic needs of the developing fetus. In extreme cases, progressive ischemia and deposition of platelets and fibrin in the intervillous spaces may result in overt placental infarction. Further investigation will be required to determine the mechanisms by which trophoblast-reactive antibodies inhibit the binding of uPA to its receptor; it is possible that antibodies with specificity for the glycolipid anchor, which mediates attachment of the uPA receptor protein to the cell surface, may be present in these patients.

Identification of broadly reactive antitrophoblast antibodies adds one more consideration to other well-described immunologic causes of fetal loss. Although this study was confined to women with ACAI, it is possible that trophoblast-reactive antibodies may also occur in women with unexplained fetal loss, even in the absence of ACAI. Therefore, further characterization of antitrophoblast antibodies may provide additional insight into the pathogenesis of immunologically mediated fetal loss, and the management of women presenting with this disorder.

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Detection of antitrophoblast antibodies in the sera of patients with anticardiolipin antibodies and fetal loss

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