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Antigens of human trophoblasts: A working hypothesis for their role in normal and abnormal pregnancies

(placentas/immunity/oncofetal/membranes)

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ABSTRACT **This report describes the preparation and characterization of antisera to human trophoblast membranes. Rabbit antisera were raised to trophoblast microvilli prepared by differential ultracentrifugation. Antibodies to serum proteins were removed by solid-phase immunoabsorption with normal human serum, and indirect immunofluorescence experiments with cryostat sections of human placentas showed that the absorbed anti-trophoblast sera reacted with trophoblasts as well as with stromal cells and endothelium of chorionic villi. The antisera also produced membrane fluorescence when studied on viable lymphocytes and certain human cell lines. These anti-trophoblast sera were also lymphocytotoxic, and this reaction was abolished by prior absorption of the antisera with leukocytes. The leukocyte-absorbed anti-trophoblast sera retained their ability to react with trophoblasts and certain human cell lines, but no longer reacted with lymphocytes or placental stromal cells and endothelium. Two categories of trophoblast membrane antigens are thus defined: one present on trophoblasts and certain human cell lines (tentatively designated TA_1), and the other on trophoblasts and lymphocytes, villous fibroblasts, and endothelium (tentatively designated TA_2). A working hypothesis is proposed stating that normal pregnancy involves the generation of anti-TA_2 subsequent to blastocyst implantation and entrance of trophoblasts into the maternal circulation. This involves a mechanism similar to allogeneic cell stimulation and results in antibodies that block either the recognition or cytotoxicity of TA_1. Failure to mount this response allows TA_1 recognition and trophoblast immunopathology. Experimental and clinical studies in support of this working hypothesis, particularly involving abortion and toxemia, are cited from published reports.**

Several provocative ideas have been put forward suggesting that the mechanisms responsible for the integrity of the trophoblast homograft are essentially immunological in nature (1). In humans the functional interface between mother and fetus is the trophoblast (2), and immune reactions occurring on this membrane would seem to be central to the well being of the graft. Detailed investigation of trophoblast antigens is thus a necessary prerequisite to an understanding of the immunology of the materno-fetal relationship, yet current information is limited almost entirely to cell products of trophoblasts such as human chorionic gonadotropins and pregnancy-associated globulins (3). As a first step in more precisely defining antigens of the trophoblast membranes, we have studied the specificity of heterologous antisera to human trophoblasts. In this report we describe evidence suggesting that human trophoblast membranes contain two major groups of antigens: one that is shared between trophoblasts and normal peripheral white blood cells, and a second group that is shared between trophoblasts and certain human cell lines. These data, together with published reports from other laboratories, make it possible to construct a working hypothesis for the role of trophoblast membrane antigens in normal and abnormal pregnancies.

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MATERIALS AND METHODS

Trophoblast membranes were prepared from freshly collected, term, normal placentas by the differential ultracentrifugation technique of Smith *et al.* (4). Pellets contained large amounts of alkaline phosphatase [specific activity 0.89 with *p*-nitrophenyl phosphate as substrate (5)], indicating the presence of trophoblast membranes (6). Twelve outbred Old English rabbits were injected intramuscularly with 2 mg of the membrane preparation in 2.5 ml of Freund's complete adjuvant containing 1 mg of *Mycobacterium tuberculosis* per ml, boosted 6 weeks later with a further 2 mg interperitoneally, and bled after 12 days. Each rabbit received membrane preparations from a separate, normal placenta. The antisera were assayed for anti-β_2-microglobulin activity as an additional check for the purity of membranes used as antigens since we and others have previously reported (7–9) that, in contrast to cells of the villous stroma, human trophoblasts lack β_2-microglobulin. None of the antisera contained anti-β_2-microglobulin activity, as measured by radioimmunoassay.

The antisera raised against trophoblast pellets contained precipitating antibodies to several serum proteins, particularly IgG, transferrin, and α-2-macroglobulin, as measured by immunoelectrophoresis against pooled normal human serum and Ouchterlony gel diffusion against IgG, transferrin, and α-2-macroglobulin. To remove these activities we passed each antiserum through a column of Sepharose-bound normal human serum (10) and then heat-inactivated each one (56° for 30 min) and absorbed each with two volumes of pooled, washed, and packed human erythrocytes. After these procedures, antisera were negative by both immunoelectrophoresis and gel diffusion against normal human serum, IgG, transferrin, and α-2-macroglobulin and are hereafter referred to as anti-trophoblast sera. The fluorescein isothiocyanate (FITC) conjugate (Wellcome Reagents, Beckman, Kent, England) of sheep anti-rabbit Ig used for immunofluorescence microscopy was also chromatographed over an immunoabsorbent column of Sepharose-bound normal human serum to remove heterophilic antibodies, and both anti-trophoblast sera and FITC-conjugates were ultracentrifuged at 100,000 × *g* every week to remove IgG aggregates which are capable (11) of reacting with Fc receptors of fetal stem vessel endothelium as well as with mononuclear blood cells.

Abbreviations: FITC, fluorescein isothiocyanate; P_i/NaCl, phosphate-buffered saline.

Tissue and Cell Membrane Immunohistology. Central cotyledon tissues from fresh, normal human placentas were snap-frozen in 2-methylbutane that had been cooled with liquid nitrogen, and 4-μm sections were prepared with a cryostat. Sections were washed 1 hr in pH 7.2 phosphate-buffered saline (P_i/NaCl) at 5° and reacted with dilutions of normal rabbit sera and anti-trophoblast sera at room temperature for 20 min. The slides were washed twice in an excess of P_iNaCl for 15 min per wash, reacted for 20 min with the FITC conjugate sera of sheep anti-rabbit Ig, washed three times in P_i/NaCl and mounted in 50% glycerol in pH 8.6 P_iNaCl. Preliminary titration experiments according to Faulk and Johnson (12) indicated that anti-trophoblast sera could be used at 1:150 dilution and conjugate at 1:60. The preparations were then examined by transmitted light from an HBO-50 mercury arc lamp for immunofluorescence with a Zeiss Universal microscope fitted with an FITC interference primary filter, Tiyoda dark ground condenser with toric lens, a 520 barrier filter, and a 40× planapochromatic objective. Blocking and absorption controls were done according to Faulk and Hijmans (13). Photographs were made with Ektachrome film.

For experiments involving cell suspensions, 5×10^5 of human cell lines HeLa, AV_3, or Hep-2, or by the normal human peripheral blood mononuclear cells prepared by the Ficoll–Isopaque technique (14) were reacted for 20 min with increasing dilutions of normal rabbit control sera or anti-trophoblast sera in Eagle's minimal essential medium containing 3% fetal calf serum. The cells were then washed twice in medium and reacted for 20 min with serial dilutions of the FITC sheep anti-rabbit Ig. After the cells were washed three times they were suspended in 50% glycerol in P_i/NaCl and examined by epi-illumination for membrane immunofluorescence with a Leitz orthoplan microscope fitted with an FITC dichoric mirror and a 54× planapochromatic objective. Preliminary titration experiments indicated that membrane immunofluorescence experiments could be satisfactorily performed with anti-trophoblast sera at 1:100 dilution and its conjugate at 1:30.

Cytotoxicity Experiments. Ten microliters of human peripheral blood mononuclear cells at 2×10^6/ml was added to an equal volume of double serial dilutions of control or anti-trophoblast sera and incubated at room temperature for 1 hr, after which 20 μl of rabbit complement (Buxted Rabbit Co., Buxted, England) was added before incubation at 37° for 30 min. Cells were removed with a micropipette and cytotoxicity was measured by counting cells in a hemocytometer after addition of 0.1% trypan blue.

Absorption Experiments. Anti-trophoblast sera were diluted 1:10 in P_i/NaCl absorbed 6–11 times with packed leukocytes. Cells for absorption were prepared from human blood in citrate by mixing with an equal volume of 3% gelatin in P_i/NaCl and sedimenting at 37°. Supernatant cells were washed, counted, and packed at a concentration of 50×10^6 leukocytes per tube immediately before absorption. Absorptions were done at room temperature for 40 min with mixing. This procedure was repeated until the cells used for absorption were negative for rabbit Ig by membrane immunofluorescence. In other absorption experiments, ultracentrifuge-prepared trophoblast microvilli pellets were used. More recently, absorption-control experiments have been done with ultracentrifuge-prepared trophoblast pellets extracted with hypertonic salt and solubilized in nonionic detergent. For this, pellets were extracted for 12 hr in 3 M KCl to remove plasma membrane or plasma proteins, and the KCl-washed material was solubilized in 1.0% deoxycholate according to Faulk *et al.* (15). The KCl-extracted, detergent-solubilized trophoblast cell membranes were dialyzed against 0.15 M carbonate buffer (pH 7.8)/P_i/NaCl, containing 0.1% deoxycholate and 10 mM sodium azide, at room temperature and adjusted to a protein concentration of 0.5 mg/ml.

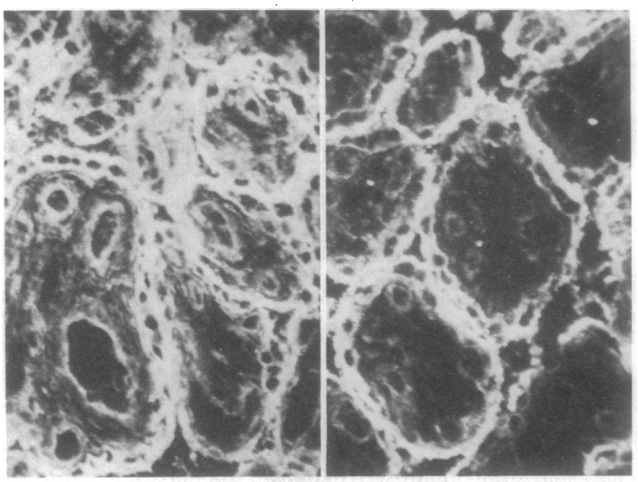

FIG. 1. Immunostaining of human placenta with (*Left*) anti-trophoblast serum and (*Right*) leukocyte-absorbed anti-trophoblast serum. Note staining of trophoblasts, placental vessel endothelium, and cells of the villous stroma with anti-trophoblast serum (*Left*) and staining reactions limited to trophoblasts with leukocyte-absorbed anti-trophoblast serum (*Right*). (×324.)

RESULTS

Specificity of Anti-Trophoblast Sera for Trophoblasts. Each of the 12 anti-trophoblast sera was studied for trophoblast specificity by indirect immunofluorescence of 25 normal human plancentas. Brilliant fluorescence of trophoblasts was produced by all antisera (Fig. 1 *left*), and titration of individual antisera showed little variation of different placentas, suggesting that there was no type specificity. Staining of fetal stem vessel endothelium and some cells of the villous stroma was also observed (Fig. 1 *left*).

The specificity of trophoblast staining by anti-trophoblast sera was tested by the principle of antigen absorption control according to McCormick *et al.* (16), using KCl-extracted, detergent-solubilized trophoblast cell membranes. Volume-for-volume absorptions of these 12 antisera were done with solubilized trophoblast microvilli for 1 hr at 4° followed by ultracentrifugation for 1 hr at 100,000 × *g* in a Beckman model L5-75 ultracentrifuge to remove immune complexes. The absorbed antisera stained neither trophoblasts nor any of the cellular components seen in Fig. 1 *left*. In addition, the antisera absorbed with membrane preparations from 15 different placentas failed to stain any aspects of chorionic villi, suggesting no individual or type specificity.

Specificity and Cytotoxicity of Anti-Trophoblast Sera for Other Cells. The anti-trophoblast sera were studied for other membrane specificities by epi-illumination immunofluorescence using normal peripheral blood mononuclear cells and human amnion (AV_3), Hep-2, and HeLa cells. The human cell lines stained much more intensely than the peripheral blood mononuclear cells with anti-trophoblast sera (Fig. 2 *right*), and, in all experiments, greater than 95% of the cells were positive. As with the above results using trophoblasts, staining of mononuclear cells, AV_3, Hep-2, and HeLa cells was removed by absorption of anti-trophoblast sera with either intact ultracentrifuge-prepared trophoblast microvilli or with detergent-solubilized trophoblast cell membranes.

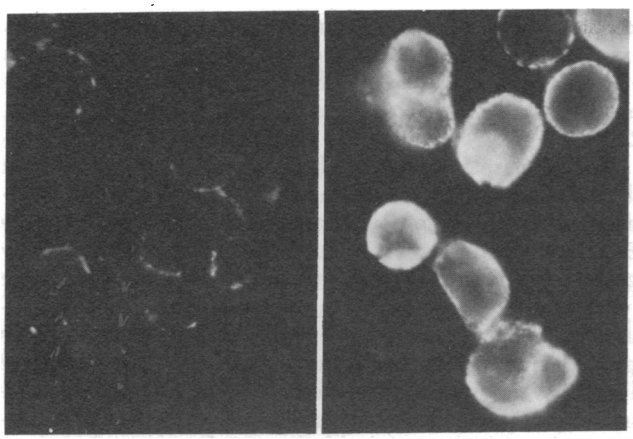

FIG. 2. Immunostaining of (*Left*) peripheral blood mononuclear cells and (*Right*) Hep-2 cells with anti-trophoblast serum. Note difference in intensity of staining of anti-trophoblast serum for blood mononuclear and Hep-2 cells. (×400.) HeLa and AV_3 cells were similarly stained, although HeLa cell staining was removed by leukocyte absorption (×20) of the antiserum.

Since the rabbit anti-human trophoblast membrane sera contained antibodies reacting with cell membranes of peripheral mononuclear cells, we also studied their cytotoxic effect. All antisera were cytotoxic for mononuclear cells from 10 different cell donors (Fig. 3). The least cytotoxic antiserum caused a loss of viability of 30% of the cells at 1:800 dilution; the most cytotoxic antiserum caused a 30% viability at 1:6000. The end-point for each was within two double serial dilutions for all 10 donors, suggesting that these antisera contained no specificities for individual HLA antigens.

In order to test the specificity of cytotoxicity of anti-trophoblast sera for human peripheral blood mononuclear cells, the anti-trophoblast sera were absorbed with human peripheral leukocytes. After 11 absorptions, none of the antitrophoblast sera were more cytotoxic than normal (nonimmune) rabbit serum, even at titers as low as 1:30 (Fig. 3). Indeed, some of the anti-trophoblast sera lost lymphocytotoxicity activity after as few as six absorptions with leukocytes (Fig. 3).

Immunohistological Specificity of Leukocyte-Absorbed Anti-Trophoblast Sera. Although leukocyte-absorbed anti-trophoblast sera were no longer cytotoxic for peripheral blood mononuclear cells, upon examination of cryostat sections of placentas it was found that as many as 20 absorptions with leukocytes did not noticeably diminish the intensity of trophoblast staining (Fig. 1 *right*), although staining of cells in the mesenchymal stroma and the endothelial staining of fetal stem vessels in chorionic villi was removed. This pattern of immunostaining was not affected either by absorption of the antiserum with human placental akaline phosphatase (Sigma Chemicals, St. Louis) or by blocking experiments using unlabeled antisera to human chorionic gonadotropin (National Institute of Biological Standards, Holly Hill, England) or to the placental glycoproteins SP_1 and PP_5 (kindly supplied by Behringwerke AG, Marburg, Germany). No other human tissues, including cryostat sections of liver, kidney, lung, spleen, brain, pituitary, ovary, testes, pancreas, stomach, and small and large bowel, were stained with the anti-trophoblast sera after leukocyte absorption. In contrast, the leukocyte-absorbed (×20) anti-trophoblast sera caused brilliant membrane fluorescence of a population (25–40%) of AV_3 and Hep-2 cells. Continued absorption of these reagents with leukocytes did not cause a further loss of AV_3 or Hep-2 cell staining, indicating that absorption was complete with 20 absorptions. Thus, in summary, trophoblast antigens, as studied by the specificity of heterologous anti-trophoblast sera, can be classified into two major

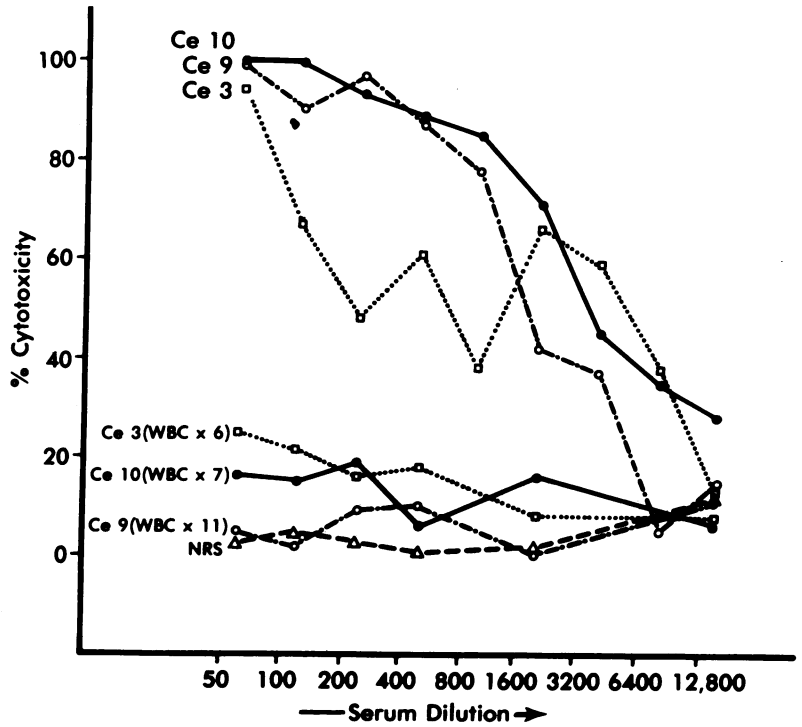

FIG. 3. Cytotoxicity and absorption experiments of anti-trophoblast serum for peripheral blood mononuclear cells. Different anti-trophoblast sera are designated by Ce. Cytotoxicity experiments are shown in the upper three lines. Lower three lines plus normal rabbit serum (NRS) are the same anti-trophoblast sera after leukocyte (WBC) absorption the designated number of times. Note that after leukocyte absorption the anti-trophoblast sera were no more cytotoxic for blood mononuclear cells than was normal rabbit serum.

groups: one that is shared between trophoblasts and certain human cell lines, and one that is shared between trophoblasts and normal human peripheral blood mononuclear cells.

DISCUSSION

We propose that the group of antigens that are sheared between trophoblasts (Fig. 1 *right*) and human cell lines (Fig. 2 *right*) be tentatively designated as TA_1 (trophoblast antigens, 1) and that antisera to these antigens be described as anti-TA_1. Similarly, we propose that the group of antigens responsible for the specificity of anti-trophoblast sera for peripheral blood mononuclear cells (Fig. 2 *left*), leukocytes (Fig. 3), and placental vessel endothelium (Fig. 1 *left*) be tentatively designated at TA_2 (trophoblast antigens, 2), and that antibodies to these antigens be described as anti-TA_2. This grouping of human trophoblast antigens as TA_1 and TA_2 can be used to interpret the result of an important previous investigation done in rats. Beer *et al.* (17) reported that heterologous antisera to rat trophoblasts cause abortion in pregnant rats as well as death in some of the pregnant mothers. However, when the antisera were absorbed with rat lymphocytes, the investigators observed only abortion without maternal death. According to our proposed nomenclature, it would appear that lymphocyte absorption removed anti-TA_2, thus eliminating the serum's ability to recognize maternal cells and leaving the oncofetal TA_1 specificity, which mediated immune damage to trophoblasts causing abortion without maternal death.

From our data we should like to suggest that normal human pregnancy involves a maternal immune response to the lymphocyte crossreactive trophoblast antigen, TA_2. Such antibodies would be expected to bind to TA_2 present in normal placentas. Support for this comes from two lines of evidence: First, antibodies of maternal origin that recognize and block certain lymphocyte functions can be eluted from placentas of both mice (18) and humans (19), and, second, similar yet more specific blocking antibodies of lymphocyte function have been identified in maternal sera (20). Even though these antibodies block certain lymphocyte responses *in vitro*, their function *in vivo* is unestablished. It is, however, interesting that mothers who lack *in vitro* evidence of serum blocking factors do tend to sustain repeated abortions (20). An absence of blocking function is thus suggestive of the absence of anti-TA_2.

In light of the above classification of trophoblast antigens, we wish to put forward a working hypothesis concerning the immunobiology of normal pregnancy that is relevant to the immunopathology of abnormal pregnancies. One traditionally assumes that mothers are "tolerant" to the placental homograft. We suggest that tolerance, if abortion is to be averted, is the lack of an effective response to TA_1. This can theoretically be accomplished by mounting an antibody response to TA_2 which inhibits either recognition of or cytotoxicity to TA_1. This could operationally be done in a hapten–carrier system where the lymphocyte crossreactive TA_2 (interpreted by mother as "self" antigen) is the hapten and the oncofetal trophoblast TA_1 (interpreted by mother as "foreign") antigen is the carrier. This would explain the presence of an antibody with crossreactive specificity for antigens on host tissues, but has the marked theoretical disadvantage of possibly generating cell-mediated immunity to TA_1. However, the need for cooperation of carrier T cells in a hapten–carrier system can be circumvented by allogeneic cell stimulation (21), providing that antigen-reactive B cells are present in the recipient (22, 23) and that a graft-versus-host reaction be induced (21, 22). Successful implantation and growth of the blastocyst is a type of graft-versus-host reaction, trophoblast tumors being the most extreme type of this example, and it is well documented that proliferation of the embryo and placenta are accompanied by the passage of trophoblasts (allogeneic cells) into the mother's circulation (24, 25).

There is ample information from normal subjects to support the presence of antigen-binding B lymphocytes for "self" antigens in conditions of low-zone tolerance, such as shown by Bankhurst *et al.* for thyroglobulin (26), and we have recently produced evidence for the presence of trophoblast-reactive lymphocytotoxic antibodies in the sera of pregnant and nulliparous subjects with systemic lupus erythematosus (27). The pregnant mother is thus endowed with antigen-reactive B lymphocytes for trophoblast antigens, and she receives allogeneic membranes (trophoblasts) subsequent to the implantation and proliferation of the blastocyst in her uterus. In light of these observations, we suggest that the production of anti-TA_2 in normal pregnancy results from allogeneic cell stimulation of maternal TA_2 antigen-reactive cells by trophoblast membranes. We presently envision that these antibodies function to block either the recognition or cytotoxicity of TA_1, but the role of helper and supressor cells in this reaction has yet to be established.

This mechanism for the immunobiology of normal pregnancy could fail if the allogeneic effect failed, resulting in recognition of TA_1 and abortion. Confirmation of this potentially immunopathological effect is drawn from a recent report by Komlos *et al.* (28) that abortions are more frequent in couples who share common HL-A antigens. Shared histocompatibility antigens would diminish the possibility of allogeneic cell stimulation of TA_2-reactive maternal B cells, thus allowing TA_1 recognition and abortion. This concept could also apply to other conditions of abnormal pregnancies. For example, Jenkins *et al.* (29) have reported that in toxemia there is a greater degree of HLA compatibility between couples and Feeney *et al.* have reported that toxemia is much less common in women who have received a blood transfusion before becoming pregnant (30). The observation of increased spontaneous abortion in HLA compatible couples is consistent with the above hypothesis of failed allogeneic helper effect, and, in the blood transfusion studies, we would suggest that TA_2 and histoincompatible leukocyte antigens in the blood transfusion primed the recipients for more effective allogeneic recognition during pregnancy. By the same token, toxemia without prior transfusion could serve to abrogate allogeneic recognition in subsequent pregnancies, accounting for the more frequent appearance of toxemia in the first pregnancy.

In closing, it should be emphasized that we do not suggest that toxemia is associated with particular HLA types [notwithstanding increased homozygosity as suggested by Redman and Bodmer (31), which is quite another matter], and such associations have not indeed been shown to exist (32), although the degree of parental HLA compatibility was not studied (32). Trophoblast antigens such as TA_1 and TA_2 are however coded by the embryo and, although most known antigens of the major histocompatibility complex are absent from trophoblasts (9), it is not unlikely that HLA compatibility between the mother and father will be associated with materno-fetal TA_2 compatibility, thus deminishing the possibility of maternal anti-TA_2, and thereby allowing TA_1 recognition and disease. These ideas will no doubt change as knowledge of trophoblast antigens expands, but in their present state they do seem to form a working hypothesis which draws support both from our own experimental work as well as from the published reports of others.

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