ANTIGENS OF HUMAN TROPHOBLAST
Effects of Heterologous Anti-Trophoblast Sera
on Lymphocyte Responses In Vitro*

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An attractive, though unproven, explanation for the apparent lack of rejection of allogeneic fetal tissues by the mother is that components of the placenta normally serve to modulate maternal immunity (1). Evidence in support of a central role for the placenta in the host-parasite relationship of human pregnancy comes from several lines of research. For instance, immunohistological investigations of human placentae have revealed the presence of IgG and complement within chorionic villi (2) and immunogenetical studies have shown that placental IgG is of both maternal and fetal origin (3). IgG eluted from placentae demonstrates specificity for homologous trophoblast basement membranes (4, 5) indicating the presence of placental-specific antigens, and such eluates have been shown to inhibit lymphocyte blastogenic responses to tuberculin, phytohemagglutinin, and allogeneic lymphocytes (6). This inhibition is apparently caused by antibody, because F (ab′)2 fragments of placental IgG continue to inhibit, and Gm typing of eluted IgG indicates that it is predominately maternal (6, 7). This suggests that mothers mount vigorous immune responses to fetal antigens during normal pregnancies and that evidence of these responses can be identified in normal placentae. However, the nature of the antigens involved is largely unknown.

To define the placental antigens to which mothers might respond, we have focused our attention on the trophoblast, the cell which constitutes the operational interface of the host-parasite relationship during human pregnancy. Results of previous studies published from this laboratory (8) have resolved the specificity of heterologous antisera to intact and fractionated trophoblast cell membranes into two major antigen groups: one being common to several human tissues including normal peripheral blood lymphocytes (PBL)1 and the other shared only between trophoblasts and certain human cell lines maintained in vitro. Because large numbers of trophoblasts are reported to pass into the maternal circulation during normal pregnancy (9) it is

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1 Abbreviations used in this paper: anti-TA, antisera to unfractionated trophoblast membranes; FITC, fluorescein-isothiocyanate; ID, immunodiffusion; IEP, immunoelectrophoresis; MLC, mixed lymphocyte culture; NHS, normal human serum; NRS, normal rabbit serum; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline.
reasonable to suspect that mothers might mount specific recognition to these allogeneic fetal membranes. Although human trophoblasts lack all known antigens of the major histocompatibility complex (10-12), studies with rodent blastocysts have shown that certain of their minor histocompatibility antigens can be serologically identified on trophoblast (13). This indicates that maternal lymphocytes encounter allogeneic trophoblast antigens, but neither the nature nor the biological function of these antigens have been studied. We have thus investigated the effects of heterologous antitrophoblast sera on lymphocyte responses to allogeneic cell stimulation.

Materials and Methods

Antisera. Placentae from normal pregnancies were collected from the delivery theater within 15 min of birth. Trophoblast microvilli prepared by differential ultracentrifugation (14) contained large amounts of alkaline phosphatase (15) and exhibited microvilli by transmission electron microscopy. They lacked histocompatibility antigens and β2-microglobulin as determined by histocompatibility testing, inhibition of hemagglutination, and radioimmunoassay (12), thereby indicating negligible contamination by membranes from other placental cells. Trophoblast membranes were washed overnight in 3 M KCl, solubilized in 1% deoxycholate, and chromatographed through a column of Bio-Gel P-200 (Bio-Rad Laboratories, Richmond, Calif.) (16). Rabbits were injected with 2 mg each of pelleted trophoblast membranes or with 1.2 mg of the first peak (known hereafter as peak 1) from Bio-Gel columns. All immunizations included 2.5 ml Freund's complete adjuvant containing 1 mg/ml M tuberculosis, and were boosted intraperitoneally 1 mo later with an additional 2 mg of alum-precipitated antigen according to Ploom (17), and the animals were bled a fortnight later. The antisera raised against pelleted trophoblast membranes contained precipitating antibodies to IgG, transferrin, and alpha-2-macroglobulin as measured by immunoelectrophoresis (IEP) against pooled normal human serum (NHS), and in double-radial immunodiffusion (ID) against the purified proteins. These antibodies were removed by solid-phase immunoabsorption employing a column of Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.)-bound NHS (18) until the sera were negative against NHS in IEP and ID.

All antisera were heat inactivated (56°C for 30 min) and absorbed with 2 vol of pooled, washed, packed human erythrocytes (RBC). Antisera to pelleted trophoblast membranes and peak 1 were additionally absorbed twice with PBS (phosphate-buffered saline)-washed lyophilized human liver powder, and twice with Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) prepared PBL. The PBL absorptions were done by mixing 1 × 10⁸ cells in 3 ml of undiluted sera for 40 min at room temperature. In addition, 1 g of lyophilized human liver powder washed three times in PBS and packed by ultracentrifugation at 100,000 g for 20 min was mixed with 3 ml of PBL-absorbed antisera for 1 h at 4°C followed by ultracentrifugation at 100,000 g for 1 h, and the supernate pipetted and aliquoted into 0.5-ml sterile plastic containers and kept at −70°C until use.

After liver-PBL absorptions, both antisera failed to react with PBL membranes by either membrane immunofluorescence or cytotoxicity. Following the tentative nomenclature proposed for a group of human trophoblast antigens in a previous publication (8), the liver-PBL absorbed antiserum to pelleted, unfractionated trophoblast membranes is hereafter referred to as anti-TA₁, and the antiserum to solubilized, fractionated trophoblast membranes is referred to as anti-peak 1. Control hyperimmune sera were obtained from four rabbits that had been immunized with frozen-thawed X3, washed HEp-2 cell membranes and absorbed and processed exactly as the anti-TA₁ and anti-peak 1 sera. Additional control (nonimmune) sera were obtained from a pool of 10 unimmunized healthy rabbits. All sera used in this study were ultracentrifuged at 100,000 g 4°C for 60 min before use to remove protein aggregates and immune complexes.

Cells. Blood was collected in acid-citrate-dextrose solution and mononuclear leukocytes isolated by Ficoll-Hypaque gradient centrifugation, washed and resuspended to 2.5 × 10⁶ cell/ml in RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 100 mg/ml streptomycin and 15% pooled AB human plasma as previously described (19). Stimulator cells
received 3,000 rads in a \(^{137}\)cesium irradiator (M-38-3 Gammator; Isomedix Inc., Parsippany, N. J.) to abolish their capacity to proliferate.

**Cytotoxicity.** Microtechniques for complement-dependent cytotoxicity have been described in detail (20). Briefly, 1.0 µl of antisera were incubated at room temperature for 60 min with 1.0 µl of cell suspension (2.5 \(\times\) \(10^6\)/ml). After one wash, 2.0 µl of rabbit complement were added. After an additional 30-min incubation at 37°C, trypan blue in 2% EDTA was added and the viability assessed.

**Lymphocyte Cultures.** To determine the effect of the heterologous antisera in MLC, 250,000 responder cells (0.1 ml) were cultured with 250,000 stimulator cells (0.1 ml) in flat bottom microtiter trays (Linbro Chemical Company, Hamden, Conn.). Cultures were prepared in quadruplicate and incubated in an air-5% CO\(_2\) incubator for 5 d at 37°C. \(^{3}\text{H}\) thymidine (Schwartz/Mann Div., Becton, Dickinson, & Co., Orangeburg, N. Y.) was added (2 µCi/well) and the cells harvested 18 h later in a multiple sample harvester (MASH II, Microbiological Associates, Walkersville, Md.).

**Immunofluorescence.** For experiments involving cell suspensions, 5 \(\times\) \(10^5\) human PBL, isolated as above, were reacted for 20 min with a predetermined optimal dilution of normal rabbit sera or anti-trophoblast sera in RPMI-1640 containing 3% fetal calf serum. The cells were washed twice in medium and reacted for 20 min with 1:30 dilution of fluorescein-isothiocyanate (FITC) conjugated sheep anti-rabbit IgG (Burroughs Wellcome Co., Research Triangle Park, N. C.). After three washes the cells were suspended in 50% glycerol in PBS and examined by epillumination for membrane immunofluorescence with a Leitz orthoplan microscope (Leitz/Opto-Metric Industrial Div. of E. Leitz Inc., Rockleigh, N. J.) fitted with an FITC dichromatic mirror and a 54X planapochromatic objective. Preliminary titrations as done in other trophoblast studies (21) indicated that membrane immunofluorescence experiments could be satisfactorily performed with anti-trophoblast sera at 1:90 dilution and its conjugate at 1:30.

Immunohistological reactions were done with cryostat sections of fresh placental tissues snap-frozen in liquid nitrogen-cooled isopentane. Human placentae were obtained from the Department of Obstetrics, Medical University of South Carolina, baboon (Papio papio) placenta from Dr. Vernon Stevens, Department of Obstetrics, Ohio State University, Columbus and rhesus (Macaca mulatta) placentae from Dr. Harold McClure, Department of Pathology, Emory University, Atlanta. The preparation of reagents, tissue processing and blocking and absorption controls were done according to Faulk and Hijmans (22). The FITC sheep anti-rabbit Ig was passed through an immunoadsorption column of Sepharose-bound NHS (18) to insure against the possibility that heterophilic antibodies in the conjugate might react with cryostat sections of human tissues. Assessment of reactions was done using transmitted immunofluorescence with a Zeiss universal microscope (Carl Zeiss, Inc., N. Y.) employing a 75-W xenon light source, FITC primary interference filter, Tiyoda dark ground condensor fitted with a toric lens, and an interchangeable 530/500 nm barrier filter.

**Results**

**Immunofluorescence Studies.** The apical aspects of syncytiotrophoblast membranes in cryostat sections of normal term placentae reacted brilliantly with both anti-\(T\)A\(_{1}\) and anti-peak 1 at 1:200 dilution, whereas no other tissues of the chorionic villi reacted with these reagents (Fig. 1). In addition, neither antiserum reacted with monkey or baboon placentae, even when employed undiluted. Unstimulated peripheral blood lymphocytes from 30 different donors were studied by membrane fluorescence with anti-\(T\)A\(_{1}\) and anti-peak 1 by using the same PBL-liver absorbed antisera that produced the intense immunofluorescence of human trophoblasts seen in Fig. 1. The positive control serum in these experiments was unabsorbed antiserum to pelleted trophoblast membranes, and the negative control sera were trophoblast absorbed anti-trophoblast serum and normal rabbit serum (NRS). In all instances, 100% of the cells reacted with the unabsorbed positive control antiserum to pelleted trophoblast membranes at 1:150 dilution whereas none of the cells reacted with anti-\(T\)A\(_{1}\), anti-
peak 1, trophoblast-absorbed anti-trophoblast serum, or NRS, even when these reagents were used undiluted.

After the above results with unstimulated lymphocytes, allogeneically stimulated PBL from a 2-h, two-way MLC were harvested and studied for membrane immunofluorescence using anti-TA1 and anti-peak 1. The unabsorbed positive control antiserum to pelleted trophoblast membranes remained reactive with 100% of the cells, however, a small number (3-5%) of allogeneically stimulated PBL now reacted with both anti-TA1 and anti-peak 1 sera as shown by a speckled distribution of immunofluorescence on their membranes, but control reactions with trophoblast-absorbed anti-trophoblast serum and NRS at the same or lower dilutions remained negative. In addition, dose-response experiments, with the use of lower dilutions of anti-TA1 and anti-peak 1 sera, increased the intensity of membrane fluorescence with lymphocytes from MLC reactions but had no effect on the total percentage of positive cells. Lymphocytes from control cultures, e.g., JJr in Fig. 2, were nonreactive by membrane fluorescence with both anti-TA1 and anti-peak 1.

The specificity of these immunohistological reactions was monitored with positive and negative control sera, and all reagents employed in this aspect of the study were chromatographed through columns of insolubilized NHS to remove heterophilic antibodies, and were ultracentrifuged to remove possible immune complexes and aggregates. The observation that PBL-liver absorbed anti-trophoblast sera react with the membranes of allogeneically stimulated PBL and not with unstimulated cells...
suggests that trophoblast cross-reactive antigens appear on lymphocytes as a result of either the recognition or response to allogeneic membranes. The specificity of this reaction is further supported by our previous report that such trophoblast cross-reactive antigens do not appear on the cell membranes of lectin-stimulated lymphocytes (23).

**Effect of Anti-Trophoblast Sera on MLC Reactions.** Several experiments were designed to study the biological effects of anti-trophoblast and control sera on in vitro lymphocyte functions. Anti-TA1 and anti-peak 1 sera at 1:50 final dilutions were 90% inhibitory in the MLC reaction, whereas NRS and anti-HEp-2 were either stimulatory or without effect (Fig. 2). This MLC inhibition was reproducible using lymphocyte combinations from at least 10 different donors suggesting that these antisera are not capable of recognizing allotypic differences. We have previously shown that preincubation of anti-peak 1 with either stimulator or responder cells has no suppressive effect on MLC reactions (23). These results indicate that trophoblast cross-reactive lymphocyte antigens appear on allogeneically stimulated lymphocytes and that antibody to these antigens can significantly inhibit the MLC reactions. Dose-response studies showed that [\(^{3}\)H]-thymidine uptake was totally inhibited by increasing the concentration of anti-peak 1 (Fig. 3), and parallel studies of cell viability using trypan blue exclusion showed that the presence of anti-TA1 and anti-peak 1 sera at all concentrations in Fig. 3 were not cytotoxic. Control cultures with NRS and anti-HEp-2 serum at identical concentrations were without effect, thus making most unlikely the possibility that suppression was produced nonspecifically.

To further characterize the suppressive properties of anti-peak 1, kinetic and absorption studies were undertaken. In one set of experiments where anti-peak 1 was added to different MLC reactions at hourly intervals, maximal suppression (85%) was observed between the 1st and 6th h of culture (Fig. 4). Addition of anti-peak 1 to MLC reactions after the cells have been in contact for >18 h had little or no
FIG. 3. Total inhibition of MLC is obtained by increasing concentrations of anti-peak 1. Each bar represents the average of quadruplicate cultures ± SEM.

FIG. 4. Time-course experiments showing peak inhibition by anti-peak 1 at 2-6 h and little suppressive effect after 18 h.

suppressive effect. No suppression was observed in parallel control experiments where NRS or anti-HEp-2 sera were added hourly during the initial 24 h of culture. Thus, we conclude that trophoblast cross-reactive lymphocyte antigens are transiently found on lymphocyte membranes, with optimal expression occurring between 1 and 6 h...
after an allogeneic stimulus. Specificity and control studies of MLC inhibition by anti-trophoblast sera showed that absorption with trophoblast membranes removed anti-peak 1 suppression whereas absorptions with HEp-2 cells or PBL had no effect (Fig. 5). These observations provide further support for the absence of trophoblast cross-reactive lymphocyte antigens on unstimulated lymphocytes.

To test the biological and species specificity of anti-peak 1 serum we conducted several experiments with sub-human primates. The effect of anti-peak 1 on monkey lymphocytes showed that both the human response to rhesus lymphocytes and the rhesus response to human lymphocytes were inhibited by anti-peak 1 (Fig. 6), whereas addition of NRS was without effect. Thus, stimulated rhesus lymphocytes may also express trophoblast cross-reactive lymphocyte antigens that are similar to those found in humans or that anti-peak 1 serum is equally effective in suppressing both stimulator and responder human cells when cocultured with monkey lymphocytes (Fig. 6). In contrast, anti-peak 1 caused no inhibition in [3H]-thymidine incorporation in baboon: baboon reactions nor in MLC where baboon cells were responding to human lymphocytes (Fig. 7). However, positive control experiments showed that the baboon lymphocytes used for our studies underwent vigorous MLC reactions when exposed to irradiated lymphocytes from either humans or unrelated baboons. Furthermore, the addition of anti-peak 1 was not significantly different from NRS, presumably indicating the lack of human-like trophoblast cross-reactive lymphocyte antigens on baboon lymphocytes, suggesting that the MHC of rhesus lymphocytes may be more closely allied to human than to baboon MHC. Similarly, significant increases in
Rhesus:human MLC responses are completely abolished by anti-peak 1. Bars represent the average of quadruplicate cultures ± SEM.

Baboon:baboon and baboon:human MLC reactions are not inhibited by anti-peak 1. Bars represent the average of quadruplicate cultures ± SEM.

$[^3]H$ thymidine incorporation were observed when human lymphocytes were stimulated by irradiated baboon cells in culture and this was not inhibited by anti-peak 1 sera (Fig. 8). Such augmentation of the MLC in the presence of anti-trophoblast sera was never seen in human allogeneic combinations, thus, it is possible that either a
different recognition mechanism exists for human-baboon interactions, or that anti-trophoblast sera react differently in this system.

Discussion

The phenomenon of antibody enhancement has been invoked by several investigators as a possible explanation for survival of allogeneic trophoblast in the maternal uterus, and a certain amount of evidence has been put forward to support this hypothesis (6, 24, 25). A large number of reports have shown blocking factors during pregnancy which inhibit lymphokine, mitogen, antigen, and MLC responsiveness, and some of these have shown that blocking activity copurifies with the IgG fraction of serum or placental eluates (6). It is thus an attractive possibility that antigens responsible for these activities are trophoblast membrane components, especially when one considers that the trophoblast membrane forms the operational interface between the mother and fetus for all mammals with hemochorial placentae. However, it is very difficult to interpret any of the above reports in terms of TA₁ or peak 1 antigens, because we have previously shown that neither anti-TA₁ nor anti-peak 1 interfere with lymphokine or mitogen responses (23). Furthermore, it is not presently known whether the mother's immune system is even capable of responding to these fetal antigens, and if she were, it it not apparent whether her responses would benefit or destroy the trophoblast. Finally, it would seem that histocompatibility-dependent reactions to presently known MHC antigens are largely irrelevant to human trophoblast as they are not present on these cells (10–12).

The precise nature of TA₁ is not presently clear. We have shown in a previous publication that peak 1 contains a group of glycoproteins that resolve into at least
four bands in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (16). We know that neither anti-TA1 nor anti-peak 1 bind labeled human placental lactogen, human chorionic gonadotropin, follicle-stimulating hormone, or the placental glycoproteins PP5 and SP1 by radioimmunoassay, although radio-labeled peak 1 is bound by both antisera (W. P. Faulk, V. Stevens, and J. A. McIntyre. 1978. Unpublished observations). Although peak 1 contains alkaline phosphatase activity (16), neither anti-peak 1 nor anti-TA1 manifest immunohistological, immunocytological, or complement-mediated cytotoxicity effects similar to those achieved with rabbit antisera to human placental alkaline phosphatase, suggesting that the biological effects of our anti-trophoblast sera on lymphocytes are not a result of antibodies with specificity for placental alkaline phosphatase. Immunohistological studies with anti-TA1 and anti-peak 1 in our laboratory have shown that these reagents do not react with cryostat sections of any other normal human tissue except syncytiotrophoblast, and similar studies have shown that neither antisera reacts with trophoblasts in cryostat sections of baboon or rhesus placentae. Although these species-specific antisera fail to react with other normal tissues, they do however recognize the cell membranes of certain human transformed cell lines maintained in vitro (26, 8). It thus seems as though trophoblasts contain a group of species- and organ-specific antigens which we have tentatively designated as TA1 (8), and that closely related trophoblast cross-reactive antigens are found on allogeneically stimulated lymphocytes and on certain lines of human transformed cells.

That lymphocyte TA1 is truly a trophoblast cross-reactive antigen is shown by our monkey experiments. Although neither anti-TA1 nor anti-peak 1 reacts with monkey placentae, they are potent inhibitors of rhesus:human MLC reactions, whether the rhesus lymphocytes serve as stimulator or responder cells. This observation is compatible with an earlier report by Revillard et al. (27) which demonstrated that human placental eluate prolonged rhesus skin graft survival as well as inhibited monkey MLC and blastogeneic responses to PHA. Unlike placental eluates, neither anti-TA1 nor anti-peak 1 inhibit lymphocyte responses to lectins (23), suggesting that trophoblast cross-reactive antigens of human lymphocytes are products of specific cell-cell recognition and do not occur simply as a result of either cell culture or mitogen stimulation. Evidence in support of this specificity is drawn from the effects of anti-peak 1 on baboon: baboon and baboon:human MLC reactions. Although baboon: baboon combinations produce strong MLC reactions, these are not inhibited by human anti-trophoblast sera. In this instance it appears as though human trophoblast cross-reactive lymphocyte antigens are not present on MLC-stimulated baboon PBL. If human trophoblast cross-reactive antigens fail to appear on lymphocytes subsequent to stimulation, as in the case of baboon:human MLC reactions, it is puzzling that anti-trophoblast sera then serve as potent stimulators of cellular proliferation. Perhaps stimulation rather than suppression occurs if specific recognition of trophoblast cross-reactive lymphocyte antigens is incomplete or does not occur between responder and stimulator cells. We presently do not know if anti-trophoblast serum is affecting stimulator or responder cells. However, our data showing inhibition of MLC by anti-trophoblast serum several hours after culture initiation implicates an effect of antibody on responder cells, but the requirement for stimulator cells to be alive (28) suggests

\[ \text{Faulk, W. P., C. Yeager, J. A. McIntyre, and M. Ueda. 1978. Human trophoblast antigens in cellular transformation and the host-parasite relationship of pregnancy. Submitted for publication.} \]
that they may also express trophoblast cross-reactive antigens, a process which we assume requires metabolic activity and thus cell viability.

Our interpretation of these results is that human trophoblast contain antigens not found on other normal tissues, including unstimulated PBL. This is confirmed by the observation that selectively-absorbed antisera to either pelleted trophoblast membranes or to solubilized and fractionated trophoblast are neither cytotoxic for lymphocytes nor do they react with cells or tissues other than trophoblasts. Because anti-TA1 and anti-peak 1 are potent inhibitors of MLC reactions, it would follow that reaction products on lymphocytes are made available subsequent to MLC. This is confirmed by the observation that anti-peak 1 reacts with lymphocyte membranes shortly after initiation of MLC, although the same cells are nonreactive before encountering allogeneic stimulation. Our impression that these newly appearing reactive-sites are trophoblast cross-reactive lymphocyte antigens is further strengthened by the observation that they are found on human:rhesus MLC-stimulated lymphocytes although they are absent from the rhesus placenta. In view of the reported absence of MHC antigens on human trophoblast (10-12), it is unlikely that the sites which appear after MLC are classical histocompatibility antigens, however, their failure to appear after stimulation by baboon lymphocytes suggests that they are associated with allogeneic recognition. Trophoblast cross-reactive lymphocyte antigens thus seem to be reaction products of cell-cell interactions, and the nature of these antigens appears to be determined by the specificity of the recognition signals which initiate the reaction. The role of these antigens in the immunobiology of the host-parasite relationship of human pregnancy is largely unexplored. Their role in the biology of lymphocyte responses to allogeneic cells may also provide new approaches to the study of genetically determined immunological recognition and responsiveness.

Summary

This report describes the inhibition of human mixed lymphocyte culture (MLC) reactions by rabbit antisera to intact and detergent solubilized, fractionated, human trophoblast membranes. Heat-inactivated antisera were passed through solid-phase immunoadsorption columns of normal human serum and extensively absorbed with human erythrocytes, lymphocytes and liver powder. Immunohistological experiments with these absorbed antisera showed that they reacted brilliantly with syncytiotrophoblast in cryostat sections of human but not baboon or monkey placentae, and not with other normal adult tissues including peripheral blood lymphocytes (PBL). Addition of these antisera to MLC reactions produced significant and reproducible suppression of responses without affecting cell viability. Absorption studies demonstrated complete removal of MLC inhibition with trophoblast membranes but not with PBL or suspensions of HEp-2 cells. Timed experiments showed that optimal inhibition occurred when the antisera were added between 2 and 6 h after culture initiation, and that little suppression was achieved after 18 h. Lymphocytes harvested from MLC reactions after 2 h showed that 3-5% of the cells reacted with PBL/liver-absorbed anti-trophoblast sera, and that unstimulated PBL were negative. Cultures of subhuman primate lymphocytes in the presence of heterologous antisera to human trophoblast membranes showed total inhibition of rhesus:human and human:rhesus MLC, and no suppression of baboon:human or human:baboon reactions, whereas human lymphocytes responded in an exaggerated manner when stimulated by baboon
cells. Modulated MLC responses to human, rhesus, or baboon lymphocytes, in the presence of anti-trophoblast sera indicate that the antisera recognize trophoblast cross-reactive lymphocyte antigens. We propose that these antigens are reaction products of cell-cell interactions, and that the nature of the antigens is determined by the specificity of the recognition signals which initiate the reaction.

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ANTIGENS OF HUMAN TROPHOBLAST

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