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Immunology

Trophoblast modulation of maternal allogeneic recognition

(placenta/immunity/suppression/membranes/lymphocyte)

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ABSTRACT **Human syncytiotrophoblast cell membranes prepared by differential ultracentrifugation were extracted with 3 M KCl, solubilized in 1% deoxycholate, and chromatographically separated into two peaks by passage through a column of Bio-Gel P-200. Previous reports from this laboratory have shown that the first peak (P1) is serologically the same as a group of trophoblast membrane antigens tentatively designated as TA_1. Microgram amounts of P1 protein were found to completely inhibit the mixed lymphocyte culture (MLC) reaction but had no suppressive effect on lymphocyte responses to the lectins phytohemagglutinin or pokeweed mitogen. Control P1 membrane fractions identically prepared from human erythrocytes and liver powder had no inhibitory effects on either MLC reactions or lymphocyte responses to mitogens. Dose–response experiments with P1 from 10 different placentae showed total inhibition of MLC by all preparations when used between 25 and 50 μg/0.2-ml MLC mixture, but some P1 fractions inhibited significantly at much lower concentrations. Timed experiments revealed that MLC suppression was maximal when P1 was added within 12 hr after culture initiation and that no effect could be found with addition after 48 hr. We have previously shown that TA_1 is a lymphocyte product of allogeneic responses, and the present results indicate that P1 proteins are themselves involved in the biology of lymphocyte responses to allogeneic cells. Pregnancy is one of the few natural circumstances in which a mixing of allogeneic cells occurs *in vivo*, and the presence of P1 proteins at the operational interface in the host–parasite relationship of human pregnancy suggests that this trophoblast membrane constituent is involved in the modulation of maternal allogeneic responses.**

Portions of the blastocyst that develop into the human embryo are sequestered from contact with maternal tissues by extraembryonic cells of fetal origin that, at maturity, are recognized as the amnion and placenta (1). Much of the amnionic membrane is contiguous with maternal uterine decidua (2), and the placenta is in contact with circumfluent maternal blood, thus providing for both anatomical contact and physiological exchange between genetically dissimilar tissues. In other circumstances of transplantation this would normally incite immunological rejection, yet the amnion and placental homografts grow unimpeded. Protection of the fetus from such rejection seems to be dependent on certain properties of the extraembryonic membranes, because mothers normally mount vigorous immunological responses to fetal allotypically incompatible antigens that leak free of these membranes into the maternal circulation (3–5).

It is not presently known how extraembryonic tissues protect the embryo and fetus from damaging immunological reactions, but contemporary research suggests that more than one mechanism may be involved. For instance, syncytiotrophoblasts lack all presently known antigens of the major histocompatibility complex (MHC) (6–8), thus denying the opportunity for conventional allogeneic recognition. Another unusual property of extraembryonic cells is the presence of membrane receptors for transferrin (9); invertebrates such as schistosomes have long employed binding of host proteins to escape immunosurveillance (10). And finally, blocking antibodies have also been implicated in the transplantation immunobiology of the host–parasite relationship of human pregnancy (11, 12).

Previous studies from this laboratory (13) with the use of heterologous anti-trophoblast sera have shown that trophoblast cell membrane antigens can be classified into at least two major groups, which we have tentatively designated as trophoblast antigens 1 (TA_1), and trophoblast antigen 2 (TA_2). The TA_1 group is organ and species specific (14) and is identified on allogeneically stimulated but not on either resting or mitogen-stimulated lymphotocytes (15). Antisera raised to a chromatographically isolated trophoblast membrane fraction, known as peak 1 (P1), have been shown to contain anti-TA_1 activity, and these antisera are able to inhibit the mixed lymphocyte culture (MLC) reaction (15, 16), suggesting that trophoblast cross reactive lymphocyte antigens play a role in the immunobiology of lymphocyte responses to allogeneic cells. Because pregnancy is the only natural occurrence in which there is a mixing of allogeneic cells, we have studied the effects of trophoblast membrane fractions on the MLC reaction. In this report we present the results of our studies of human syncytiotrophoblasts, showing that the membranes of these cells contain proteins that specifically inhibit allogeneic recognition without disrupting other lymphocyte functions.

MATERIALS AND METHODS

Trophoblast Membrane Preparations. Trophoblast microvilli were prepared from 10 placentae delivered of uncomplicated pregnancies by normal mothers and were collected and processed within 15 min of delivery. The organ was minced with scissors into 1- to 2-cm^3 pieces and microvilli were prepared from these tissues according to the differential ultracentrifugation technique of Smith *et al.* (17). The exclusive presence of microvilli in the ultracentrifuged pellet was confirmed by transmission electron microscropy. These microvilli contained large amounts of alkaline phosphatase (13), suggesting that they were harvested from trophoblasts (13, 18), and they lacked both HLA antigens and β_2-microglobulin as determined by histocompatibility testing (19) and radioimmunoassay (8), thus indicating negligible contamination of the pellet with membranes from other placental cells. The microvilli were extracted by using a constant-motion rotor for 16 hr at 15°C in an excess of 3 M KCl to remove peripheral proteins, washed twice in an excess of phosphate-buffered 0.15 M saline (P_i/NaCl) at pH 7.4, and solubilized in 1% deoxycholate in 0.2

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Abbreviations: MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; P_i/NaCl, phosphate-buffered saline; PHA, phytohemagglutinin; PWM, pokeweed mitogen.

† Present address is the same as that for W.P.F.

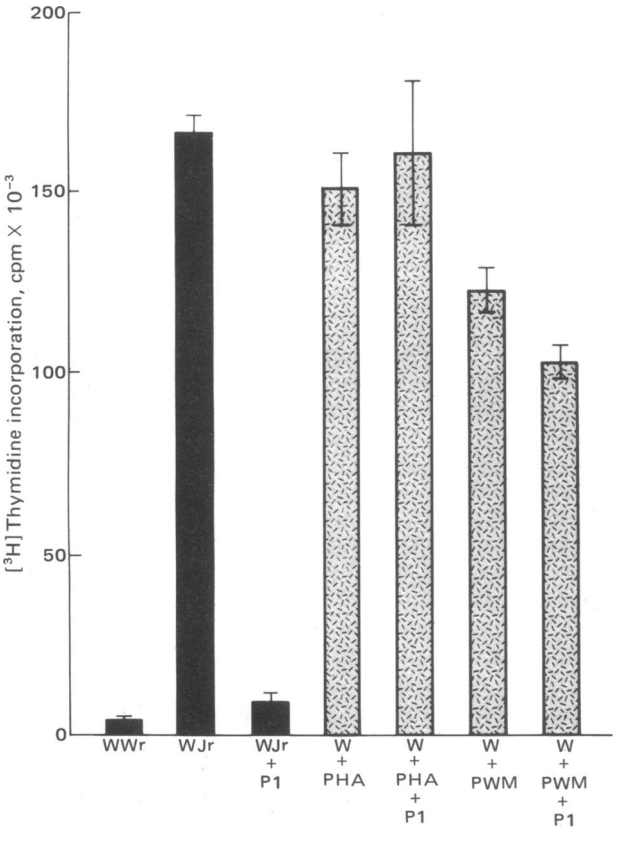

FIG. 1. Inhibition of MLC reactions between cells from individuals W and J by P1 trophoblast membrane proteins. r, Irradiated cells. Each bar represents the average of triplicate cultures; error lines show SEM. Note that concomitant addition of P1 to mitogen-stimulted cultures of W's cells was without significant effect.

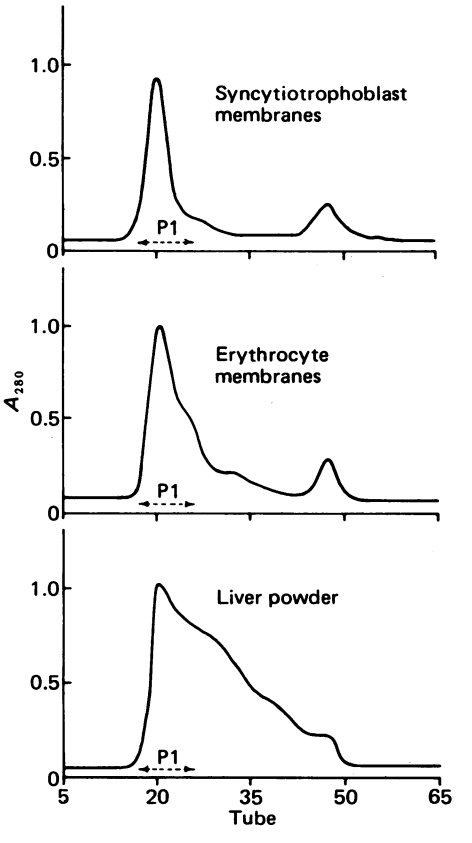

FIG. 2. Bio-Gel P-200 chromatography: tracings of the protein contents of solubilized human syncytiotrophoblast, erythrocyte, and liver membranes. P1 fractions from each membrane preparation were inclusive of tubes 17–26.

M sodium carbonate/bicarbonate buffer at pH 10 for 24 hr. Insoluble material was removed by centrifugation at 250 × *g* and the supernatant was fractionated into two peaks by chromatography through a 2.5 × 25 cm column of Bio-Gel P-200 according to the technique of Faulk *et al.* (18). The first peak (hereafter known as P1) from this separation was extensively dialyzed against P_i/NaCl, concentrated by vacuum dialysis, ultracentrifuged at 100,000 × *g* for 1 hr at 4°C to remove aggregates, and diluted to 1.0 mg/ml with sterile P_i/NaCl. Previous reports from this laboratory have shown that the P1 from trophoblast membranes is serologically the same as a group of trophoblast membrane antigens tentatively designated TA_1 (13). P1 preparations were also made from pooled human erythrocytes and P_i/NaCl-washed, lyophilized, human liver powder according to the same techniques, and all preparations were subjected to irradiation with 15,000 rads (150 grays) in a ^{137}Cs irradiator (M-38-3 Gammator, Isomedix Inc., Parsippany, NJ) for sterilization before introduction into MLC reactions.

Cells. Peripheral blood samples were collected in citric acid/sodium citrate/dextrose (ACD) solution and mononuclear leukocytes were separated by Ficoll/Hypaque gradient centrifugation, washed, and resuspended to 2.5 × 10^6 cells per ml in RPMI 1640 medium (GIBCO) containing 100 mg of streptomycin per ml and 15% pooled AB human plasma as described (20). Stimulator cells received 3300 rads (33 grays) in the ^{137}Cs irradiator to abolish their capacity to proliferate.

Lymphocyte Cultures. To study the biological effect of the trophoblast and control membrane preparations in MLC reactions, 250,000 responder cells (0.1 ml) were cultured with 250,000 stimulator cells (0.1 ml) in flat-bottom microtiter trays (Falcon 3040). The lyophilized mitogens phytohemagglutinin (PHA, GIBCO) and pokeweed mitogen (PWM, GIBCO) were reconstituted and 0.025-ml samples of previously determined optimal stimulatory dilutions were added to cell cultures containing only responder cells. Cultures were plated in triplicate and incubated in an air/5% CO_2 incubator for 5 days at 37°C. [^{3}H]Thymidine (Schwarz/Mann) was added [2 μCi per well (1 Ci = 3.7 × 10^{10} becquerels)] and the cells were harvested 18 hr later in a multiple automated sample harvester (MASH II, Microbiological Associates, Walkersville, MD).

RESULTS

Several experiments were conducted to study the biological effects of trophoblast and control P1 membrane proteins on lymphocyte responses *in vitro*. The addition of 25 μg of P1 was sufficient to abolish all MLC reactivity (Fig. 1). Identical amounts of P1 had no significant effect upon lymphocyte responses to previously determined optimal stimulatory doses of PHA or PWM (Fig. 1). From these data, we conclude that the addition of microgram amounts of P1 can cause total suppression of allogeneic recognition in MLC reactions but has a negligible effect on lymphocyte responses to mitogens, suggesting that the biological mechanisms employed by lymphocytes in responses to mitogens are different from those to allogeneic cells.

To eliminate the likelihood of nonspecific suppression due to membrane solubilization or TA_1 preparation, MLCs were exposed to identical concentrations of P1 membrane proteins prepared from both human erythrocytes and liver tissue (Fig.

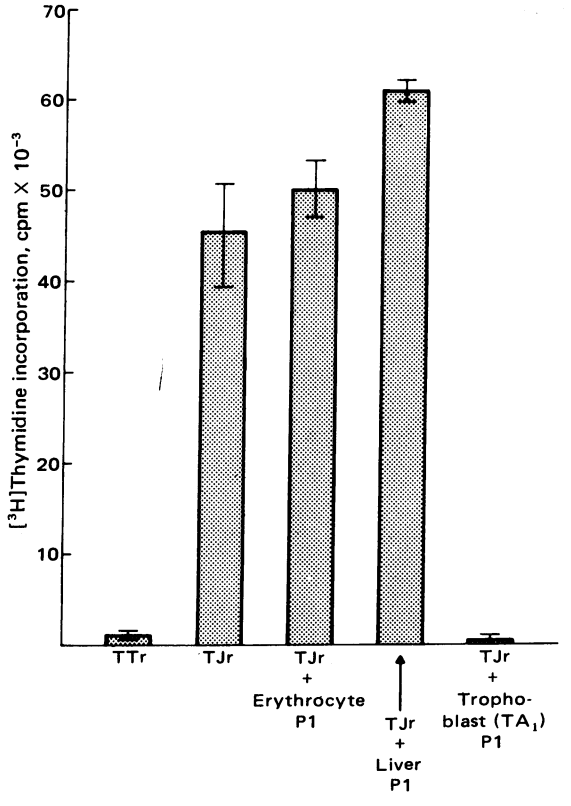

FIG. 3. MLC reactions of cells from individuals T and J; r, irradiated cells. Control P1 membrane preparations from human erythrocytes and liver tissue were either without effect or slightly stimulatory in MLC, whereas trophoblast P1 abolished MLC reactivity. Each bar represents the average of triplicate cultures ± SEM.

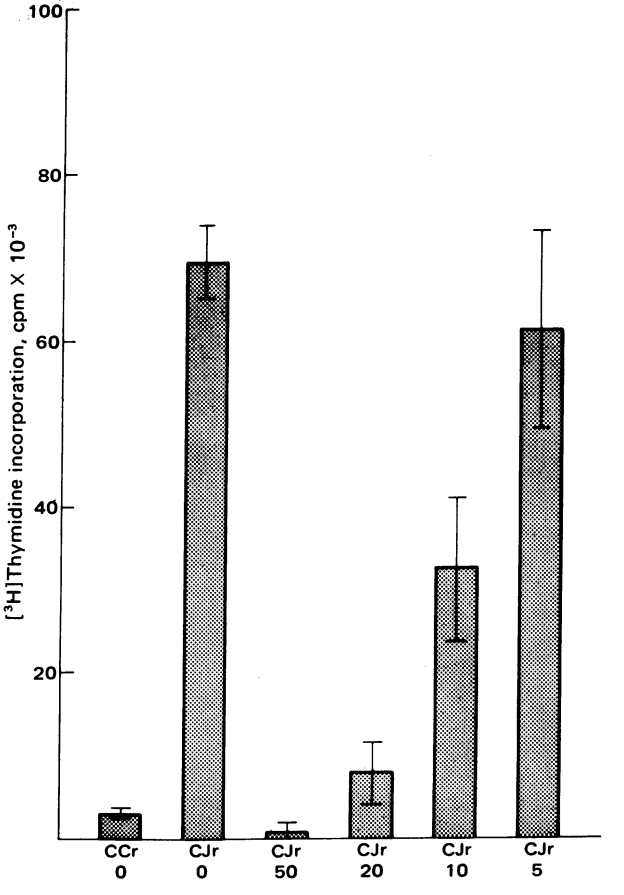

FIG. 4. Dose–response studies showing decreased suppression of MLC upon P1 dilution. The MLC included cells from individuals C and J; r, irradiated cells. The numbers below each bar indicate μg of P1 added. Each bar represents the average of triplicate cultures ± SEM.

2). Trypan blue exclusion assays indicated that neither trophoblast nor control P1 preparations from erythrocytes or liver were cytotoxic. Addition of P1 cell membrane control proteins either was without affect or enhanced MLC (Fig. 3), thus providing direct evidence that suppression is uniquely associated with trophoblast membranes. We have previously published indirect evidence in support of this concept, showing inhibition of MLC reactions with heterologous anti-P1 sera (15).

Dose–response experiments demonstrated a serial decrease in inhibition after dilution of P1 with culture media (Fig. 4). There was, however, a slight variation in the suppressive ability of each P1 preparation, presumably representing technical variations in the isolation and purification of the membrane proteins. Nevertheless, all 10 trophoblast preparations were totally suppressive when used at between 25 and 50 μg. Indeed, in one instance, significant suppression was achieved with 4 μg (not shown). Timed studies showed P1 inhibition of MLC to be maximal when P1 was added some time during the first 12 hr of culture (Fig. 5). Susceptibility to inhibition began to decline by 18 hr and was absent after 48 hr. These observations provide evidence that the suppressive effect of P1 on allogeneically stimulated lymphocytes depends on the time of administration, being undetectable if the P1 is added to an MLC after 48 hr of culture.

DISCUSSION

In this report we have shown that human trophoblast membrane proteins recovered on the first peak (P1) of chaotrope-extracted, deoxycholate-solubilized, chromatographed trophoblast membranes are responsible for inhibiting allogeneic recognition reactions without altering lymphocyte responses to mitogens. P1 fractions isolated from control erythrocytes and liver tissue were ineffective when used in identical concentrations and culture conditions. Although the nature and biological mechanism responsible for trophoblast–lymphocyte interactions are unknown at present, evidence that sites for P1 or P1-like molecules become available on lymphocyte mem-

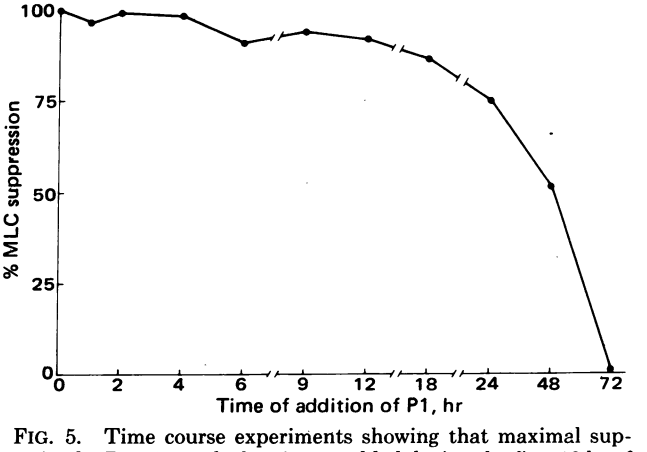

FIG. 5. Time course experiments showing that maximal suppression by P1 occurred when it was added during the first 12 hr of culture. By 18 hr susceptibility to suppression began to diminish and after 48 hr it was absent.

branes subsequent to allogeneic stimulation is shown both by immunofluorescence (16) and by anti-P1 inhibition of MLC reactions (15). We have now shown that inhibition of MLCs by trophoblast membrane proteins is also both dose and time dependent, and that maximal suppression occurs during the first 12 hr of culture and disappears after 48 hr. It is presently unclear why both P1 and anti-P1 should impede allogeneic responses, but a rather similar phenomenon is found with antisera to insulin receptors: anti-receptor antibody and insulin have the same metabolic effects (21, 22). Whether a P1 receptor is involved in the reactions we describe is not known, but the data do indicate that allogeneic stimulation either produces or exposes trophoblast crossreactive lymphocyte antigens that are involved in the biology of allogeneic reactions.

Mammalian lymphocytes are uncommonly required to express *in vivo* the phenomenon that *in vitro* is known as allogeneic recognition. However, during gestation, MHC-lacking syncytiotrophoblast membranes break away from chorionic villi and enter the maternal circulation via the uterine veins (23). Taylor and Hancock (24) have provided *in vitro* data indicating that maternal lymphocytes are capable of mounting cytotoxic responses to these membranes, but the sensitizing antigens responsible for generating these reactions are not known. If maternal recognition of trophoblast antigens *in vivo* were potentially detrimental to the trophoblast, the placental bed is the logical anatomical site where such reactions must be modulated to ensure the integrity of the extraembryonic membranes. It thus seems more than coincidence that the membranes that form the operational interface between the mother and fetus in the host–parasite relationship of human pregnancy are exactly the cells that produce the MLC-inhibiting TA_1 proteins. The concentration of trophoblast membranes in maternal blood is greatest within the intervillous spaces, and is progressively diluted as the blood passes into the uterine veins and inferior vena cava, thus creating a physiological circumstance in which maternal allogeneic reactions are inhibited only at the maternofetal interface. Our present data indicate that these trophoblast membrane proteins are effective inhibitors of allogeneic reactions *in vitro* and prompt us to suggest that the gene(s) responsible for this property has been amplified in trophoblasts through natural selection as placentation has evolved in mammals.

1. Boyd, J. D. & Hamilton, W. J. (1970) *The Human Placenta* (Heffer & Sons, Cambridge, England).
2. Hoyes, A. D. (1975) *Obstet. Gynecol. Ann.* **4,** 1–38.
3. Jeannet, M., Werner, C., Ramirez, E., Vassalli, P. & Faulk, W. P. (1977) *Transplant. Proc.* **9,** 1417–1422.
4. Levine, P. (1976) *Rev. Fr. Transfus. Immunohematol.* **19,** 213–299.
5. Faulk, W. P., van Loghem, E. & Stickler, G. B. (1974) *Am. J. Med.* **56,** 393–396.
6. Faulk, W. P. & Temple, A. (1976) *Nature (London)* **262,** 799–802.
7. Goodfellow, P. M., Barnstable, C. J., Bodmer, W. F., Snary, D. & Crumpton, M. J. (1976) *Transplantation* **22,** 595–603.
8. Faulk, W. P., Sanderson, A. & Temple, A. (1977) *Transplant. Proc.* **9,** 1379–1384.
9. Faulk, W. P. & Galbraith, G. M. P. (1979) *Proc. R. Soc. London Ser. B* **204,** 83–97.
10. Smithers, S. R., Terry, R. J. & Hockley, D. J. (1969) *Proc. R. Soc. London Ser. B* **171,** 483–494.
11. Faulk, W. P., Jeannet, M., Creighton, W. D. & Carbonara, A. (1974) *J. Clin. Invest.* **54,** 1011–1019.
12. Rocklin, R. E., Kitzmiller, J. L., Carpenter, C. B., Garovoy, M. R. & David, J. R. (1976) *N. Engl. J. Med.* **295,** 1209–1213.
13. Faulk, W. P., Temple, A., Lovins, R. E. & Smith, N. C. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1947–1951.
14. Faulk, W. P., Yeager, C., McIntyre, J. A. & Ueda, M. (1979) *Proc. R. Soc. London Ser. B,* in press.
15. McIntyre, J. A. & Faulk, W. P. (1978) *Transplant. Proc.* **10,** 919–922.
16. McIntyre, J. A. & Faulk, W. P. (1979) *J. Exp. Med.* **149,** 824–836.
17. Smith, N. C., Brush, M. G. & Luckett, S. (1974) *Nature (London)* **252,** 302–303.
18. Faulk, W. P., Lovins, R. E., Yeager, C. & Temple, A. (1977) in *Immunological Influences on Human Fertility* ed. Boettcher, B. (Academic, New York), pp. 152–160.
19. McIntyre, J. A., Turner, R. E. & LeRoy, E. C. (1978) *South. Med. J.* **71,** 1269–1279.
20. McIntyre, J. A. (1976) in *Surgical Immunology* ed. Munster, A. M. (Grune & Stratton, New York), pp, 283–306.
21. Kahn, C. R., Baird, K., Flier, J. S. & Jarrett, D. B. (1977) *J. Clin. Invest.* **60,** 1094–1106.
22. LeMarchand-Brustel, Y., Gorden, P., Flier, J. S., Kahn, C. R. & Freychet, P. (1978) *Diabetologia* **14,** 311–317.
23. Thomas, L., Douglas, G. W. & Carr, M. C. (1959) *Trans. Assoc. Am. Physicians* **72,** 140–144.
24. Taylor, P. V. & Hancock, K. W. (1975) *Immunology* **28,** 973–982.