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Human Placental Cells that Regulate Lymphocyte Function

RAOUL L. WOLF

Department of Pediatrics, Michael Reese Hospital and Medical Center and, the Committee on Immunology, Pritzker School of Medicine, University of Chicago, Chicago, Illinois 60637

ABSTRACT. We examined normal human placenta for an immunologic function by measuring the release of soluble inhibitory factor (SIF). SIF is a product of normal T lymphocytes and of the JEG-3 choriocarcinoma cell line, and blocks proliferative and antibody-producing responses of mononuclear cells. SIF can be further characterized by a noncovalently linked subcomponent, lipid suppressor substance. The villous surfaces of six normal human placentae were digested with collagenase to obtain a population of predominantly multinucleated giant cells. These cells were maintained in standard culture for 5 days after which the cell-free conditioned culture medium was assayed for SIF content by measuring suppression of [^{3}H] thymidine incorporation into lymphocytes stimulated by low-dose phytohemagglutinin. Undiluted placental SIF induced 88% inhibition of this response ($p < 0.001$). The placental SIF was found to contain lipid suppressor substance, as does SIF from mononuclear cells. We determined this by thin-layer chromatography where a peak of suppressive activity occurred at Rf 0.32 ([^{3}H]thymidine incorporation reduced from 21810 ± 308 to 4121 ± 214 cpm); this is the position on thin-layer chromatography to which mononuclear cell lipid suppressor substance migrates. Ion exchange chromatography comparing the elution patterns of lymphocyte-SIF and placental-SIF indicated that both eluted in the fraction of 40–50 mM $PO_4^{\equiv}$ buffer, further suggesting identity between these two substances. SIF from placental and lymphocyte sources functioned by inducing the presence of suppressor cells in culture. Mononuclear cells were incubated for 48 h in SIF; the resultant cell population reduced [H^3]thymidine incorporation into phytohemagglutinin-stimulated lymphocytes from 21170 ± 1721 to 8612 ± 311 cpm (60% inhibition, $p < 0.001$). Adherent cells were not required for function of these cells and they were not sensitive to mitomycin C. The placenta helps prevent immunologic rejection of the fetus by several mechanisms; it functions as a barrier, it is devoid of human histocompatibility leukocyte antigen markers and it has immunologic functions. The studies presented herein indicate that the placenta releases a substance that induces the formation of suppressor cells. These data support the hypothesis that placental cells have an immunoregulatory function. (*Pediatr Res* 23: 212–218, 1988)

Abbreviations

E_C, effector cells
FCS, fetal calf serum
[^{3}H]TdR, tritiated thymidine
HBSS, Hanks' balanced salt solution
LSS, lipid suppressor substance
MLR, mixed leukocyte reaction
MNC, mononuclear cells
PBS, phosphate-buffered saline
PHA, phytohemagglutinin
R_C, responder cells
SIF, soluble inhibitory factor
MHC, major histocompatibility complex
C:M, chloroform:methanol ratio
TLC, thin-layer chromatography
%I, percent inhibition

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Correspondence Raoul L. Wolf, M.D., Wyler Children's Hospital, Box 133, 5841 S. Maryland Avenue, Chicago, IL 60637.
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The placenta has attracted the attention of immunologists looking for an explanation for resistance of the fetal allograft to immunologic rejection by its mother. Studies exploring this topic have been reviewed by Johnson *et al.* (1). Foremost in theories explaining the phenomenon is the observation that the trophoblastic membrane appears devoid of MHC markers (2, 3) suggesting that the tissue is not recognized by maternal immune mechanisms. Tissue from uterine decidual gland epithelium also lacks maternal MHC antigens (4). Malignant and abnormal placental tissue partially express MHC markers, especially β_2 microglobulin (5, 6), suggesting that normal trophoblast actually suppresses the expression of these antigens. Other investigators have either produced evidence that the trophoblast can provoke an immune response (7) or, in contrast, is incapable of doing so and resists lysis (8). Human trophoblastic tissue continuously enters the maternal circulation and may remain sequestered within lung (9), but the significance of this event is not apparent. These trophoblastic fragments do not stimulate an immune or inflammatory response.

Several authors (10, 11) have shown that the ectoplacental cone portion of mouse placenta can inhibit some immune responses while enhancing others, indicating that the placenta can directly influence lymphocyte function. Moreover, soluble substances that stimulate immunologic responses are released by placental cells; cultures of transformed and normal trophoblastic cells release colony-stimulating factor (12), a cytokine that promotes maturation of polymorphonuclear cell cultures.

We have shown previously that the choriocarcinoma-derived cell line, JEG-3, releases SIF into culture fluid (13). SIF nonspecifically suppresses lymphocyte proliferation and antibody synthesis (14). It is released by dividing T and null cells, but not by B cells or continually growing B or T cell lines. Monocyte cultures do not generate SIF in the culture medium. SIF is distinguished by a dual structure comprising a protein moiety of 130–150 kD and a glycolipid, LSS (15). SIF has been found in normal human plasma and serum, suggesting that it is released into the circulation (16). In this study, we explored the postulate that SIF might be released by the normal human placenta, thus supporting the hypothesis that the placenta has an immunologic function.

METHODS

Placental cell cultures. Six placentae from six uncomplicated pregnancies were obtained at normal delivery within 30 min of expulsion and studied separately. Human clinical investigation committee approval was obtained for this study. The villous surface was washed with sterile saline and dissected using sterile techniques. The tissue was washed with PBS to remove blood and was cut into small pieces. Microscopic slides were prepared to note the presence of giant cells *in situ*. The slices were disrupted with collagenase (0.5%) for 30 min in a shaker water bath at 37° C to free cells. Noncellular debris was removed by filtration over gauze. The cells were washed twice in HBSS and resuspended in RPMI 1640 with added 10% heat inactivated human serum from donors AB Rh+ blood type (AB+), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (complete medium). Tissue culture flasks (25 cm^2 area) were coated with fibronectin (kindly supplied by Dr. Francois Booyse, Michael Reese Blood Bank and Research Foundation) at room temperature for 30 min. Cell suspension was added to the flasks and cells were obtained as they settled. We determined that 5 min was optimal for adherence of multinucleated giant cells. There was minimal adherence of fibroblasts and endothelial cells after 5 min. Lymphocytes and monocytes were not present in these preparations. The adherent cells were washed thoroughly to remove nonadherent cells and red blood cells and complete medium was added (10 ml/25 cm^2). Cultures were maintained at 37° C in humidified air with 5% CO_2 and were refed after 24 h. Duration of culture was 4 days, after which the conditioned media (placental SIF) were filtered (0.45 μ) and stored at 4° C until used. For some experiments, culture fluid was sampled at 24-h intervals for the total duration of culture. Media treated at 60° C for 15 min were also assayed for SIF activity. Control culture supernatant fluid was derived from smooth muscle cell and endothelial cell cultures derived from normal skin biopsy. These were kindly donated by Dr. F. Booyse and were prepared on fibronectin-coated cloning cover slips using techniques described elsewhere (17).

Preparation of lymphocyte SIF. Thirty to 40 ml of whole blood from normal healthy volunteers was diluted with an equal volume of HBSS and fractionated by density gradient over Ficoll-Hypaque (Pharmacia, Piscataway, NJ). MNC settling at the interface were washed three times in HBSS and resuspended in complete medium at 10^6 cells/ml.

SIF-containing culture fluid was prepared by MLR as described elsewhere (14). Briefly, MNC were derived from unrelated donors and each resuspended in complete medium at 5 × 10^6 cells/ml. The cell suspensions were mixed in equal volumes and cultured for 5 days in humidified air with 5% CO_2 at 37° C. The cultures were terminated by centrifuging at 900 × g for 15 min; the supernatant fluid was filtered (0.45 μ) to remove cell debris and was stored at 4° C until used.

Negative control cultures were prepared from MLR where both parties of cells were irradiated with 10,000 rad. We have previously (14) demonstrated that irradiated lymphocytes do not release SIF.

Purification of SIF and LSS. SIF was isolated by ion-exchange column chromatography using DEAE Sephadex (Pharmacia). Placental media or MLR culture fluid were dialyzed against starting buffer (10 mM PO_4^- at pH 7.6) and run into the column. SIF was eluted in a linear gradient of PO_4^- buffer from 30 to 150 mM at pH 7.0. Fractions were pooled according to the previously noted elution pattern of SIF (16). The fractions were concentrated to original volume over a membrane of nominal cutoff 10,000 daltons, dialyzed against two changes of 20× volume HBSS and one of RPMI 1640. AB+ serum (10%) was added, and the fractions were stored at 4° C until used.

LSS was derived by extraction of placental SIF and lymphocyte SIF into chloroform:methanol as described previously (15). Fifteen ml of SIF containing fluid were diluted with an equal volume of distilled water and adjusted to pH 9 with KOH-borate buffer. Thirty ml of C:M 1:1 was added slowly with mixing. The mixture was shaken vigorously and centrifuged at 6000 × *g* for 30 min to break the emulsion. The upper methanol:water phase was dried by rotary evaporation under vacuum. The residue was extracted into C:M to remove LSS from salt. Further purification was achieved by preparative TLC. Silica gel 60 plates (Scientific Products, McGaw Park, IL) were washed in C:M and allowed to dry. The LSS-containing extract was streaked in 1 cm bands at 5 μl/band. The plates were developed with C:M:water 60:38:8 to within 2.5 cm of the top. A 5 × 20 cm plate was run in parallel and charred with 50% sulfuric acid to identify bands. The mobility fraction relative to the solvent front (Rf) at 0.31–0.33 was scraped and eluted sequentially with C:M:water 15:5:1, C:M:water 5:15:1, and methanol:water 9:1. The remainder of the plate was scraped in 0.5-cm bands. The eluates were pooled, dried to remove all traces of solvent, and LSS was redissolved in complete medium and stored at 4° C until used. Control extractions were performed on control medium after passage through an ion exchange column or with solvent alone.

Assay for SIF and LSS. MNC were derived from healthy donors and resuspended in complete medium at 10^7 cells/ml. One donor was used in each of six experiments to test samples of placental SIF, lymphocyte SIF and control simultaneously. Triplicate 0.2-ml aliquots of each test and control sample were placed in 96-well microtiter plates. Dilutions of the test samples were made using complete medium. Twenty μl of cell suspension were added to each well, giving 10^5 cells/well. The cells in culture were stimulated with 20 μl of PHA at a dose (0.95 μg/ml) previously titrated by dilution curves to give 0.25 of the maximum incorporation of [^{3}H]TdR. Cultures were maintained at 37° C in humidified air with 5% CO_2 for 4 days, pulsed with [^{3}H]TdR (20 μCi/ml; 50 Ci/mol, New England Nuclear, Boston, MA) and harvested 18 h later onto filter discs using a Titertek harvester (Flow Labs, Rockville, MD). Uptake of [^{3}H]TdR into new DNA was measured by counts remaining on the filter discs using a fluor-based cocktail (RPI-3a, Research Products International, Mount Prospect, IL). LSS activity was measured with a shorter culture period of 48 h, as we have previously noted peak suppression by LSS at this time (14).

Fibroblast cultures were kindly donated by Dr. F. Booyse and were prepared as previously described (17). Once the cultures were established, the culture medium was replaced with 10 ml of complete medium on SIF-containing media. The flasks were cultured at 37% in humidified air with 5% CO_2 for 4, 16, and 72 h. The incorporation of [^{3}H]TdR into new DNA was measured after 4 h incubation.

We measured the effect of varying the concentration of PHA on the suppression induced by SIF. The volume of the SIF-containing fraction from DEAE Sephadex chromatography was kept constant. PHA was used at 0.95, 1.25, 2.0, and 2.25 μg/ml. The assay was performed as above.

Induction of suppressor cell activity. The method of Shou *et al.* (18) was modified as follows. In the first phase MNC were derived from peripheral blood of normal, healthy volunteers and suspended at 2 × 10^7 cells/ml in complete medium. Nine-tenths ml of control and test culture fluids were placed separately in duplicate wells of a 24-well cluster plate (1 cm^2 well) (Flow Labs) and 0.1 ml of cell suspension was added. The cultures were rocked at 4–6 oscillation/min in humidified air with 5% CO_2 at 37° C for 2 days. The MNC (E_C) were then washed three times in HBSS and once in RPMI and resuspended in complete medium at 10^6 cells/ml.

A titration curve of the effect of SIF containing fluids in inducing suppressor cell function was obtained by using dilutions of placental SIF and lymphocyte SIF in control media. This procedure was otherwise identical with the generation of E_C above.

Role of adherent cells. MNC, as above, were suspended at 10^7 cells/ml in HBSS with 20% heat inactivated FCS and incubated in plastic Petri dishes at 37° C in humidified air with 5% CO_2 for 45 min. Nonadherent cells were removed by aspiration and

washing of the Petri dishes with HBSS. The nonadherent cells were resuspended in HBSS with 20% FCS and the procedure repeated. Adherent cells were removed using a rubber policeman. Adherent and nonadherent cells were adjusted to a density of 2×10^7 cells/ml. The adherent cell fraction showed 95% nonspecific esterase staining (19). Nonadherent cell fraction was <0.1% positive for nonspecific esterase. Adherent cells were added to form 0, 2, 4, 8, and 10% of the total number of cells in suspension, keeping the final density at 2×10^7 cells/ml. One-tenth ml of these cell suspensions was added to 0.9 ml of placental SIF, lymphocyte SIF, or control medium and plated into 1 cm^2 wells of a 24-well cluster plate as above. Each concentration of adherent cells for each of the three groups was tested in duplicate.

Assay for suppressor cell activity. The presence of suppressor cell activity (E_C) cultures was assessed by a second phase culture to evaluate reduction of [^{3}H]TdR incorporation into fresh MNC. R_C were derived from peripheral blood of a healthy donor, unrelated to donors used in the first phase. One donor was used to assay paired samples of E_C derived in placental SIF, lymphocyte SIF, and control media. Six separate experiments were performed. These R_C were suspended in complete medium at 10^6 cells/ml and placed in microtiter wells at 10^5 cells/well. E_C from each sample of placental SIF, lymphocyte SIF, and control were added after washing three times in HBSS in ratios of E_C:R_C at 0:1, 1:1, 1:2, 1:4, 1:8, and 1:16. Each was tested in triplicate. Cultures were stimulated with PHA at a dose (0.95 μg/ml) that yielded 0.25 peak response (determined from dilution curves) and maintained at 37°C in humidified air with 5% CO_2. After 4 days, the cultures were pulsed with [^{3}H]TdR, harvested onto filter disks 18 h later, and counted for [^{3}H]TdR incorporation.

Effect of mitomycin C. In some experiments E_C were treated with mitomycin C, 50 μg/10^6 cells for 30 min before evaluation for the presence of suppressor activity. These cells were washed and assayed for comparison with E_C that were not treated with mitomycin C in paired experiments using one group of R_C for each of four experiments. Each sample was tested in triplicate. This group of experiments was performed at a ratio of E_C:R_C of 1:1.

Calculation of results. All results were expressed as mean ± SEM or as %I, where:

$$\%I = \left[1 - \frac{\text{net cpm in test}}{\text{net cpm in control}}\right] \times 100$$

Significant suppression was determined at the $p \leq 0.05$ limit by comparing paired samples of placental SIF or lymphocyte SIF and control using the paired Student's *t* test.

RESULTS

When placental villi were dissected and cells dispersed with collagenase and allowed to settle on fibronectin-treated culture flasks, we noted that multinucleated giant cells settled within 5 min, while few fibroblasts and endothelial cells were present. Lymphocytes and monocytes were not present. The multinucleated cells were firmly adherent; red cells were removed by washing the flask. Multinucleated giant cells formed 80–90% of the placental adherent cells in culture (Fig. 1). The remainder were fibroblasts (5–10%) and endothelial cells. Figure 1 consists of two parts; *A* indicates multinucleated giant cells (*arrow*) *in situ* in a cut section of trophoblastic villus and *B* shows these cells in a stained specimen of a culture of dissected and digested villous surface of a placenta. These cells are not aggregated mononuclear cells or nonomuclear giant cells, as an intact cell membrane is visible *in situ* and in culture. Moreover, the cells originate in the syntrophoblastic area of the placental villi, where multinucleated giant cells are found. Trypan blue exclusion studies demonstrated >95% viability up to 4 days of culture, with progressive fall to 50% viability by 7 days. For this reason, the cultures were terminated at 4 days.

Conditioned medium from these cultures was assayed for an effect in suppressing [^{3}H]TdR incorporation into MNC stimulated with PHA (Table 1). Undiluted placental SIF induced 98%I of [^{3}H]TdR uptake. This activity was completely removed by heating to 60° C for 15 min. These are conditions under which SIF activity is destroyed (Table 1) as we have previously demonstrated (14). Aliquots sampled at 24-h intervals demonstrated the appearance of suppressor activity (placental SIF) within 24 h of establishing placental cell cultures, with a maximal effect after 48 h (as seen in Table 1), and the suppressive effect remained constant thereafter. Neither smooth muscle cells nor skin endothelial cells released SIF into culture fluid. A dilution curve demonstrating titration of the suppressive effect of placental media is seen in Figure 2.

We evaluated the effect of placental SIF on fibroblast cultures. Baseline counts of [^{3}H]TdR uptake into dividing trophoblasts were 10210 ± 1370 cpm. This was reduced to 7311 ± 78 cpm (30%I, $n = 4$) after 4 h exposure to SIF, to 4210 ± 772 cpm (59%I, $n = 4$) after 16 h, and to 5217 ± 560 cpm after 72 h (49%I, $n = 4$).

When we varied the concentration of PHA relative to SIF, a titration effect was seen. Using 0.95 μg/ml, we obtained 89% suppression of [^{3}H]TdR incorporation (26610 ± 6101 cpm reduced to 2927 ± 1021 cpm). At 1.25 μg/ml, 50%I (13305 ± 2311 cpm) was seen and 30%I (186278 ± 3110 cpm) at 2.0 μg/ml and 28%I (19159 ± 1020 cpm) at 2.25 μg/ml.

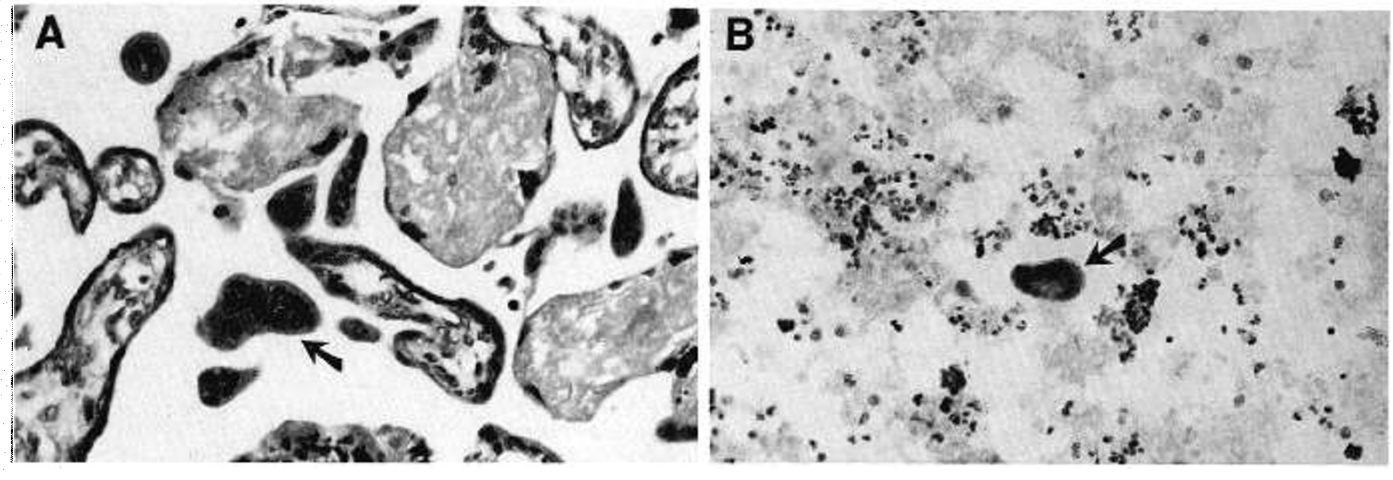

Fig. 1. *A*, a photomicrograph of a section through villous surface of the placenta. Several multinucleated giant cells are noted *in situ* (*arrow*) (original magnification ×400; Giema stain). *B*, photomicrograph of cells from the digested villous surface placenta. Multinucleated giant cells are seen (*arrow*) (original magnification, ×250; Giema stain).

Table 1. *Effect of culture media from placental cells on thymidine incorporation*

	[^{3}H]TdR-CPM*	%I
Control†	39037 ± 4516‡	
Placental-SIF§		
24 h‖	21171 ± 3101	44¶
48 h	819 ± 32	98**
72 h	670 ± 51	98**
96 h	218 ± 22	98**
Heat inactivated††	32120 ± 2180	12
Lymphocyte-SIF	3255 ± 485	92**
Heat inactivated	41012 ± 3515	0
Endothelial cell media	31112 ± 1010	13
Smooth muscle cell media	31101 ± 118	21

* Incorporation of [^{3}H]TdR into MNC stimulated with PHA and harvested after 5 days of culture.

† See text.

‡ Mean ± SEM (n = 6 experiments, each in triplicate)

§ SIF was used undiluted as generated at an original culture volume/cell ratio.

‖ Aliquots of media taken at 24-h intervals.

¶ $p < 0.05$.

** $p < 0.001$ by paired Student's t test.

†† Heat inactivation = 60° C for 15 min.

Extraction of placental-SIF into C:M and purification of this extract by TLC using silica gel yielded a fraction at Rf 0.32 that suppressed [^{3}H]TdR uptake into PHA-stimulated cells after 2 days of culture from 21810 ± 308 to 4121 ± 214 cpm (82%I, $p < 0.001$). This chromatographic pattern of suppressor activity was identical to that of LSS extracted from lymphocyte SIF (Fig. 3). No other regions of the TLC plate suppressed significantly.

MNC were incubated with control media, placental SIF, and lymphocyte SIF to assess the induction of a population of suppressor cells (E_C). These were tested on responder MNC (R_C) stimulated with PHA at a dose (0.95 μg/ml) determined from titration curves to give 0.25 of peak response. Similar experiments were performed using placental LSS and lymphocyte LSS. These results are shown in Table 2. Placental SIF and lymphocyte SIF induced the formation of E_C that suppressed significantly and were still active at a ratio of E_C:R_C 1:4. These cells were not sensitive to mitomycin C; there was no significant difference between mitomycin C-treated and nontreated groups. E_C generated in complete medium or control medium (irradiated MLR) did not suppress [^{3}H]TdR uptake into R_C. E_C generated in placental LSS or lymphocyte LSS did not suppress [^{3}H]TdR uptake into R_C.

SIF from placental and lymphocyte cultures were partly purified by ion-exchange chromatography. The results of this purification are presented in Figure 4 as %I of [^{3}H]TdR uptake into PHA-stimulated MNC. Placental SIF and lymphocyte SIF eluted in the 40–50 mM PO_4^- buffer fraction. Placental SIF in this fraction reduced [^{3}H]TdR incorporation into PHA-stimulated MNC from 27121 ± 1820 to 3210 ± 391 cpm (mean ± SEM, n = 6 experiments each in triplicate; 89%I, $p < 0.001$). Lymphocyte SIF reduced 27121 ± 1820 to 5171 ± 1002 cpm (81%I, $p < 0.001$, $n = 6$).

The SIF-containing fractions from ion-exchange chromatography of placental culture and MLR were also assessed for induction of suppressor E_C. Measured at a ratio of E_C:R_c 1:1, placental-SIF (purified) induced the formation of E_C that reduced [^{3}H]TdR incorporation from 21170 ± 1721 to 8612 ± 311 cpm (60%I, $p < 0.001$). Lymphocyte-SIF purified by ion exchange chromatography induced E_C that reduced [^{3}H]TdR incorporation from 21170 ± 1721 to 9172 ± 1100 cpm (57%I, $p < 0.001$).

We evaluated the role of adherent cells in the production of suppressor E_C. Adherent cell-depleted cultures contained fewer than 1% nonspecific esterase positive cells. These studies were

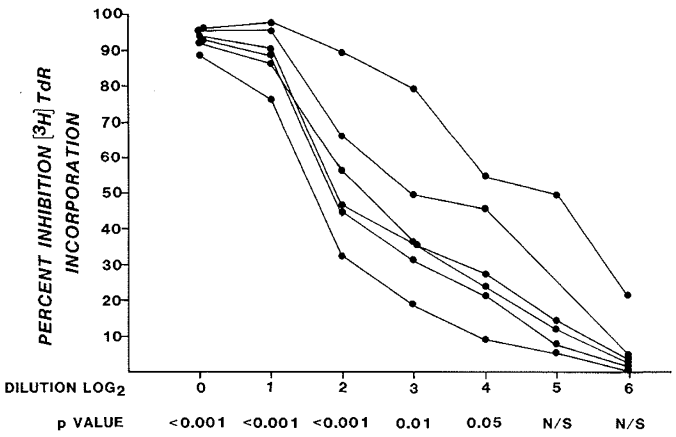

Fig. 2. Curves demonstrating titration of the suppressor effect in media of six separate placental cell cultures. Each was tested in triplicate. The X axis shows doubling dilutions of medium from placental cultures in complete medium and the Y axis depicts %I of [^{3}H]TdR incorporation into PHA-stimulated mononuclear cells. Significance of the suppression, calculated by paired Student's t test compared to control, is shown below the X axis. (*N/S*, not significant.)

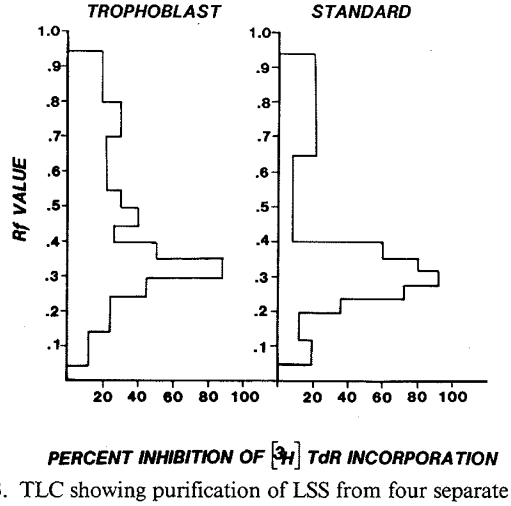

Fig. 3. TLC showing purification of LSS from four separate samples of placental-SIF. Mobility relative to the solvent front (*Rf*) is indicated on the Y axis, while %I of [^{3}H]TdR incorporation into PHA-stimulated MNC is indicated on the X axis. Significant, by paired Student's t test ($p < 0.001$) suppressor activity was found in the region Rf 0.32. For comparison, a standard preparation of LSS derived from lymphocyte SIF is shown on the right. LSS activity migrates to Rf 0.32.

performed with E_C generated in control media, placental-SIF, and lymphocyte SIF and evaluated at a ratio of E_C:R_C of 1:1 and 1:4. The results seen in Table 3 indicate that adherent cells were not required for the production of suppressor E_C in this system. Moreover, adding adherent cells at concentrations ranging from 2 to 10% of total cells did not affect the degree of suppression induced by E_C. There was no detectable difference between placental-SIF or lymphocyte-SIF in this assay. Control media did not induce E_C in the presence or absence of adherent cells.

DISCUSSION

Cultures containing 85–90% placental multinucleated giant cells were derived by dissection of the chorionic villi of normal placentae and dispersion of the cells with collagenase. This procedure yielded a mixed cell population containing mostly giant cell nests, fibroblasts, and endothelial cells. Stained sections of the microvilli of the trophoblast surface of the placenta dem-

Table 2. *Induction of suppressor cell population by placental-SIF and lymphocyte-SIF*

Ratio E_C:R_C†	CPM-(%I)* 1:1	1:2	1:4	1:8
E_C derived in				
Complete medium	28171 ± 312‡	ND§	ND	ND
Control	28341 ± 1281	23041 ± 2212	22110 ± 1370	20141 ± 1731
Placental-SIF‖	6987 ± 647 (75)¶	9517 ± 458 (59)¶	18111 ± 570 (18)	22321 ± 1152
with Mitomycin C	7213 ± 1270 (74)¶	10384 ± 1011 (55)¶	15310 ± 317 (31)**	19178 ± 1029
Lymphocyte-SIF	5447 ± 1170 (81)¶	8779 ± 943 (62)¶	16323 ± 1243 (26)**	19700 ± 1273
with mitomycin C	6116 ± 674 (79)¶	9558 ± 384 (59)¶	17243 ± 1554 (22)	22021 ± 4413
Placental-LSS	24400 ± 1201 (14)	ND	ND	ND
Lymphocyte-LSS	23113 ± 2410 (18)	ND	ND	ND
R_C alone =	24117 ± 4212 cpm (n = 12)			

* CPM of [^{3}H]TdR incorporated into PHA stimulated R_C.

† E_C were generated by incubation for 48 h in media listed. R_C were from an unrelated donor.

‡ Mean ± SEM, n = 6 experiments, each in triplicate.

§ Not done.

‖ SIF was used undiluted as generated at original culture volume/cell ratio.

¶ $p < 0.001$ by paired Student's t-test compared to control values.

** $p < 0.05$. Mitomycin C did not significantly alter suppression induced by E_C.

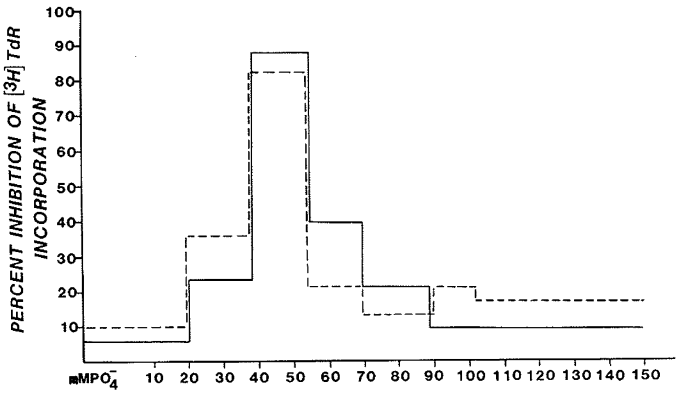

Fig. 4. Partial purification of SIF by ion-exchange chromatography over DEAE Sephadex. The X axis shows concentration of phosphate buffer (mM) and the Y axis depicts %I of [^{3}H]TdR incorporation into PHA-stimulated MNC. Absolute values are given in the text. The *solid line* represents placental and the *broken line* represents lymphocyte culture medium. SIF from both sources eluted between 40 and 50 mM PO_4-buffer.

onstrated multinucleated giant cells (Fig. 1). An intact cell membrane can be seen in Figure 1, making it unlikely that this appearance is due to cell aggregation. When these cells were allowed to settle in fibronectin-coated tissue culture flasks for 5 min, the predominant population was multinucleated cells, while fibroblasts, endothelial cells, and red cells were removed by washing the culture flasks with complete medium. For the first 4–5 days of culture, the dominant cells were multinucleated and these cells remained viable as determined by trypan blue exclusion. If any lymphocytes had in fact been present, their numbers would have been too few to contribute SIF. Unstimulated multinucleated cells released measurable suppressor activity (placental-SIF) after 24 h in culture. Aliquots of media from these cultures were taken at 24-h intervals. There was no increase in suppressor activity beyond the level generated in the first 24-h period. Release of SIF by placental giant cells without prior activation is distinct from MLR, where 5–6 days of culture are required to generate SIF (14). This time interval correlates with activation of lymphocytes by MLR Shou *et al.* (20) have demonstrated that unstimulated lymphocytes in culture produce a suppressor substance that inhibits MLR in the proliferative phase, indicating that lymphocytes can release suppressor substances without stimulation.

The suppressor activity in placental giant cell culture media presented characteristics of SIF. The activity was destroyed by heating to 60° C for 15 min, as we have noted for SIF (14). During ion-exchange chromatography of placental cell culture fluids, SIF-like activity eluted only in the range of 40–50 mM PO_4^- buffer; *i.e.* the fraction of the eluate in which SIF is found (16). SIF appears to consist of a protein noncovalently linked to LSS. The exact relationship between the protein component of SIF and LSS is not clear, however. The two molecules coelute from an ion exchange gradient after molecular seiving. Preliminary purification studies using SDS polyacrylamide gel have isolated SIF to two protein bands that migrate in the region of substances of 130,000 kD (data not shown). We have eluted LSS from this region, further suggesting that LSS forms part of the SIF molecule. The origins of the protein component of SIF and of LSS are not apparent from our data. The complete SIF molecule and LSS do not have the same range of function. Intact SIF induces a population of cells with suppressor function and suppresses both antigen- and mitogen-induced thymidine incorporation. LSS, once extracted from SIF, suppresses only PHA and does not stimulate the formation of suppressor cells. The extraction process we use to recover LSS is destructive to the protein. Thus we have been unable to evaluate the role of the protein moiety alone. It may be, however, that LSS and the protein are necessary to induce suppressor cell activity.

Others have also noted different suppressive effects by components of suppressor substances. Green *et al.* (21) described SISS, produced in response to concanavalin A. There were two components, SISS-T and SISS-B. SISS-T suppressed mitogen and antigen driven T-cell proliferation. This factor had a smaller apparent diameter than SIF, 30–45 kD. It was not cytotoxic and was unstable at 56° C, as is SIF. This suppressor substance was derived from adherent cells and mononuclear cells are required for its function, in contrast to SIF. Schnaper *et al.* (22) have shown that SIRS, produced by mouse spleen cells, can be blocked by levamisole, vitamin C, and catalase. This implies that SIRS requires granulated cells. Such cells are not necessary for SIF function.

We confirmed the presence of LSS in placental-SIF by preparative TLC. A C:M extract of placental-SIF was purified by TLC, and activity was found as a band of suppressor activity in the region of Rf 0.32 as noted previously for LSS from MLR-culture media (15). LSS appears to be a glycolipid (Wolf RL, unpublished data) that suppresses lymphocyte proliferative responses independent of the protein moiety of SIF.

Glycolipids are normal components of lymphocyte membranes (23, 24) and GM_1 is found in brain tissue and in the Thyl

Table 3. *Effect of adherent cells on generation of suppressor E_C*

	cpm [^{3}H]TdR* (%I) Concentration of adherent cells†				
	0%	2%	4%	8%	10%
E_C generated in:					
Control media					
E_C:R_C = 1:1	29002 ± 1991‡	28411 ± 1801	29211 ± 3050	20021 ± 2331	23315 ± 1001
= 1:4	30117 ± 2121	27310 ± 2002	29555 ± 2710	31510 ± 2920	26074 ± 2210
Placental-SIF§					
E_C:R_C = 1:1	6181 ± 490 (79)‖	6346 ± 1022 (78)‖	4211 ± 919 (86)‖	7221 ± 978 (64)¶	6841 ± 875 (71)‖
= 1:4	21119 ± 1302 (30)‖	19351 ± 970 (30)‖	22341 ± 1092 (35)¶	17731 ± 1921 (44)¶	18315 ± 1121 (30)¶
Lymphocyte-SIF					
E_C:R_C = 1:1	7213 ± 680 (76)‖	8317 ± 1010 (71)‖	8131 ± 1610 (73)‖	6928 ± 350 (66)‖	7871 ± 921 (67)‖
= 1:4	19721 ± 1021 (35)¶	17213 ± 991 (37)¶	20191 ± 2810 (32)¶	22217 ± 960 (30)¶	16417 ± 1221 (38)¶

* Incorporation of [^{3}H]TdR into MNC stimulated with PHA and harvested at 5 days.

† Adherent cells added or not added to MNC to generate E_C.

‡ Mean ± SEM (n = 6 experiments, each in triplicate).

§ SIF was used undiluted as generated at original culture volume/cell ratio.

‖ $p < 0.001$ by paired Student's t test comparing appropriate control with test.

¶ $p < 0.05$. No significant differences were noted when comparing different concentrations of adherent cells with each other.

marker of mouse lymphocyte membrane (25, 26). LSS may be a glycolipid common to lymphocyte and placental cell membranes which is shed into culture. McIntyre and Faulk (27, 28) have demonstrated novel alloantigens that are shared by lymphocytes and trophoblastic cells. Moreover, antibodies to these trophoblast-specific antigens suppress MLR, providing further evidence for membrane structures common to trophoblastic cells and lymphocytes. Fatty acids inserted into lymphocyte membranes alter cell function as measured by inhibition of rosette formation (29) and blocking in the migration inhibition assay (30). LSS may suppress proliferation by altering lymphocyte membrane function or fluidity. The exact source of the protein component of SIF and the mechanism through which LSS becomes associated with this molecule are not clear. SIF is released by lymphocytes but not by B cell lines (14) or endothelial or smooth muscle cell cultures, so it would appear that release of this molecule is not solely a function of cell division or cell degeneration. Moreover, lymphocytes irradiated and kept in culture alone or in irradiated mixed lymphocyte culture do not release SIF (14). SIF will suppress the incorporation of thymidine into fibroblasts as well as lymphocytes. This is a noncytotoxic mechanism. Inasmuch as SIF added just before pulsing with [^{3}H] TdR does not cause a reduction of cpm, we infer that it does not simply block thymidine but reduces proliferation and DNA synthesis. This mechanism requires further elucidation.

The placenta has recognized immunologic function. The studies of Barg *et al.* (11) demonstrated that ectoplacental cones of mice decreased T cell-dependent cytotoxicity. McIntyre and Faulk (27) found a protein from trophoblast membrane that blocks MLR, but not mitogen responses, distinguishing it functionally from SIF. While trophoblastic membrane is devoid of human histocompatibility leukocyte antigen markers (2–4), paternal antigens (H2K) are found on murine trophoblast (31) and placenta will absorb anti-H2K antibody (32). Thus, the complexities and range of the immune reactions to the placental membrane and the placental factors that modulate immune responses do not seem to have been completely defined.

We have noted that SIF added at 24-h intervals to MNC cultures stimulated with tetanus toxoid inhibited the toxoid stimulation only after 24 h of culture (16). We inferred from these data that SIF functioned via a second cell population that required at least 24 h to appear in culture. Accordingly, we studied placental SIF for an ability to produce suppressor cells. SIF induced the emergence of such a population of lymphocytes in normal MNC after 48 h in culture. The presence of suppressor cells was measured by reduction of [^{3}H]TdR incorporation into lymphocytes stimulated with a suboptimal concentration of PHA intended to produce 0.25 maximal response. The suppressor cells generated in response to SIF inhibited in a dose-dependent manner. These cells did not suppress in our assay system when the PHA was used at an optimal concentration (2.25 μg/ml). The effect could be titrated by varying the concentration of PHA implying that the effect related to the number of cells stimulated, with a greater number of cells stimulated by optimal dose PHA. The low-dose PHA responder cells may be fewer in number and thus more readily suppressed, or they may be distinct from full-dose PHA responders; our data do not distinguish these possibilities. In contrast to SIF, LSS extracted from the molecule did not induce suppressor cells. The reason for this difference is not clear, but the presence of LSS in the SIF molecule may be essential for induction of suppressor cells by SIF. Because our methods of removing LSS from SIF are destructive, we were unable to determine whether the protein component of SIF alone would induce suppressor cell activity.

We noted that the presence of adherent cells was not necessary for the induction of a suppressor cell population in response to SIF. The addition of adherent cells in concentrations ranging from 2 to 10% of total E_C did not alter the suppressor activity induced among E_C by SIF. The suppressor cells were not affected by treatment with mitomycin C, indicating that DNA synthesis was not needed for expression of suppressor function.

Suppressor lymphocytes may be instrumental in maintaining the fetal allograft. However, conflicting data have been generated regarding this role of suppressor cells. Pavia and Sites (33) did not detect an increase in suppressor cells during pregnancy, while others, using different assay systems, noted that suppressor lymphocytes in the spleen of pregnant mice were increased in comparison to nonpregnant controls (34, 35). Moreover, spleen cells of these mice will block cytotoxity (36). Smith and Sternlicht (37) have identified a population of cells in mouse spleen that are responsible for alteration of the primary response in pregnant mice. Suppressor cell activity may be detected under several circumstances, and a number of soluble substances induce the appearance of suppressor cells *in vitro*. Antigen-antibody complexes (38), interferon (39), maternal IgG (40), and concanavalin A (18) exhibit suppressor function via an intermediary suppressor cell population.

Thus, suppressive substances, such as SIF, that are released by placental cells, may induce suppressor cells *in vivo*, contributing to the maintenance of the fetal allograft.

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REFERENCES

1. Johnson PM, Brown PG, Faulk WP 1980 Immunological aspects of the human placenta. In: Finn CA (ed) Oxford Reviews of Reproductive Biology, Vol. II. Oxford University Press, Oxford, England, pp 1–40
2. Billington WD, Jenkinson J, Searle RF, Sellens MH 1977 Alloantigen expression during early embryogenesis and placental ontogeny in the mouse. Immunoperoxidase and mixed hemadsorption studies. Transplant Proc 9;1371–1377
3. Parr EL, Blanden RV, Tulsi RS 1980 Epithelium of mouse yolk sac placenta lacks H-2 complex alloantigens. J Exp Med 152:945–955
4. Johnson PM, Bulmer JN 1984 Uterine gland epithelium in human pregnancy often lacks detectable maternal MHC antigens but does express fetal trophoblast antigens. J Immunol 132:1608–1610
5. Goodfellow PN, Barnstable CJ, Bodmer WF, Snary D, Crumpton MJ 1976 Expression of HLA system antigens on placenta. Transplantation 22:595–606
6. Trowsdale J, Travers P, Bodmer WF, Patilo RA 1980 Expression of HLA-A,-B, and C and B2 microglobulin antigens in human choriocarcinoma cell lines. J Exp Med 152 (suppl):11S–17S
7. Davies M, McLaughlin EE, Sutcliffe RG 1982 Immune responsiveness against the human placenta 1. Generation of cellular and humoral activity in experimental animals. Immunology 47:459–476
8. Jenkinson EJ, Billington WP 1974 Differential susceptibility of mouse transplant and embryonic tissue to immune cell lyses. Transplantation 18:286–289
9. Thomas L, Douglas GW, Carr MC 1959 The continual migration of syncytial trophoblasts from the fetal placenta into the maternal circulation. Trans Assoc Am Phys 72:140–148
10. Pavia CS, Sites DP 1981 Trophoblast regulation of maternal-paternal lymphocyte interactions. Cell Immunol 58:202–208
11. Barg M, Burton RC, Smith JA, Luckenbach GA, Deckers J, Mitchell GF 1978 Effects of placental tissue on immunologic responses. Clin Exp Immunol 34:441–448
12. Ruscetti FW, Chou JY, Gallo RC 1982 Human trophoblasts: cellular source of colony-stimulatory activity in placental tissue. Blood 59:86–90
13. Wolf RL, Ilekis J, Benveniste R 1983 Characterization of an immune suppressor from transformed human trophoblastic JEG-3 cells. Cell Immunol 78:356–367
14. Wolf RL, Whitsed H, Rosen FS, Merler E 1978 A soluble inhibitor of B and T cell proliferation and antibody synthesis produced by dividing human T cells. Cell Immunol 36:231–241
15. Wolf RL, Merler E 1979 Role of lipids in the immune response. 1. Localization to a lipid-containing fraction of the active moiety of an inhibitor (SIF) of lymphocyte proliferation. J Immunol 123:1169–1174
16. Wolf RL, Andreoni J 1982 Soluble inhibitory factor (SIF) in normal human serum. Cell Immunol 67:299–311
17. Booyse FM, Quarfoot AJ, Chediak J, Stemerman MB, Maciag T 1981 Characterization and properties of cultured human Von Willebrand umbilical vein endothelial cells. Blood 58:788–796
18. Shou L, Schwartz SA, Good RA 1976 Suppressor cell activity after concanavalin A treatment of lymphocytes from normal donors. J Exp Med 143:1100–1110
19. Koski IR, Poplack DG, Blaese RM 1976 A nonspecific esterase stain for the identification of monocytes and macrophages. In: Bloom BR, David JR (eds) In Vitro Methods in Cell Mediated and Tumor Immunity. Academic Press, New York, pp 359–362
20. Shou L, Schwartz SA, Good RA, Peng R, Chen CL 1980 A human soluble suppressor factor affecting lymphocyte responses *in vitro*. Proc Natl Acad Sci USA 77:6096–6100
21. Greene WC, Fleisher TA, Waldman TA 1981 Soluble suppressor supernatants elaborated by concanavalin A-activated human mononuclear cells. 1. Characterization of a soluble suppressor of T cell proliferation. J Immunol 126:1185–1191
22. Schnaper HW, Pierce CW, Aune TM 1984 Identification and characterization of concanavalin A- and interferon-induced human suppressor factors: Evidence for a human equivalent of murine soluble immune response suppressor (SIRS). J Immunol 132:2429–2435
23. Schwarting GA, Summers A 1980 Gangliotetraosylceramide is a T cell differentiation antigen associated with natural cell-mediated cytotoxicity. J Immunol 124:1691–1694
24. Habu S, Kasai M, Nagai Y, Tamaoki N, Tada T, Herzenberg LA, Okumura K 1980 The glycolipid asialo GM1 as a new differentiation antigen of fetal thymocytes. J Immunol 125:2784–2788
25. Kato KP, Wang TJ, Esselman WJ 1979 Radiolabeling and isolation of Thyl active glycolipids from murine brain and lymphoma cell lines. J Immunol 123:1977–1984
26. Freimuth WC, Miller HC, Esselman EJ 1979 Soluble factors containing Thyl antigen shed from lymphoblastoid cells modulate *in vitro* plaque-forming cell response. J Immunol 123:201–208
27. McIntyre JA, Faulk WP 1979 Trophoblast modulation of maternal allogeneic recognition. Proc Natl Acad Sci USA 76:4029–4032
28. McIntyre JA, Faulk WP 1979 Antigens of human trophoblast. Effects of heterologous antitrophoblastic sera on lymphocyte responses *in vitro*. J Exp Med 149:824–836
29. Papamichail M, Tsokos G, Pepys MB, Weyman C, Belin J, Smith DA 1979 Inhibition of complement-dependent rosette formation after lymphocytic incubation with fatty acids. Immunology 38:117–122
30. Utermohlen V, Besner G, Berkowitz MG 1980 The effect of fatty acid addition *in vitro* on direct migration inhibition with paramyxoviral antigens. Clin Immunol Immunopathol 16:324–335
31. Chatterjee-Harsouni S, Lala PK 1982 Location of paternal H-2K antigens on murine trophoblast cells *in vitro*. J Exp Med 155:1679–1689
32. Wegmann TG, Mosmann TR, Carlson GA, Olijnyk O, Singh BA 1979 The ability of murine placenta to absorb monoclonal anti-fetal H2K antibody from the maternal circulation. J Immunol 123:1020–1023
33. Pavia CS, Stites DP 1979 Humoral and cellular regulation of alloimmunity in pregnancy. J Immunol 123:2194–2200
34. Chaouat G, Monnot P, Hoffmann M, Voisin GA 1982 Regulatory T cells in pregnancy VI. Evidence for T-cell mediated suppression of CTL generation toward paternal alloantigens. Cell Immunol 68:322–331
35. Suzuki K, Tomasi TB Jr 1979 Immune responses during pregnancy. Evidence of suppressor cells for splenic antibody response. J Exp Med 150:898–908
36. Clark DA, McDermott MR, Szewczuk MR 1980 Impairment of host-versus-graft reaction in pregnant mice. II. Selective suppression of cytotoxic T-cell generation correlates with soluble suppressor activity and with successful allogeneic pregnancy. Cell Immunol 52:106–118
37. Smith RN, Sternlicht M 1982 The alloantibody response in the allogeneically pregnant rat. III. The location of the alloantibody-forming cells. J Immunol 129:783–786
38. Rao US, Bennett JA, Shen FW, Gershon RK, Mitchell MS 1980 Antigen-antibody complexes generate Lyt1 inducers of suppressor cells. J Immunol 125:63–67
39. Aune TM, Pierce CW 1982 Activation of a suppressor T-cell pathway by interferon. Proc Natl Acad Sci USA 79:3808–3812
40. Harte PG, Playfair JHL 1983 Failure of malaria vaccination in mice born to immune mothers. II Induction of specific suppressor cells by maternal IgG. Clin Exp Immunol 51:157–164