TRANSCRIPTIONAL CONTROL OF HLA-A,B,C ANTIGEN IN HUMAN PLACENTAL CYTOTROPHOBLAST ISOLATED USING TROPHOBLAST- AND HLA-SPECIFIC MONOCLONAL ANTIBODIES AND THE FLUORESCENCE-ACTIVATED CELL SORTER

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Human placenta has a diversity of functions directed at maintaining the fetal environment (endocrine functions, fetomaternal nutritive, and gaseous exchange, etc.) (1). The placenta is a complex organ composed of different types of trophoblasts and a variety of other fetal tissues such as stroma, endothelium, and blood elements. Furthermore, the junctional area of the human placenta with the uterus comprises an admixture of fetal and maternally derived cells. Numerous attempts have been made to identify and characterize placental cells in single cell suspension or in primary culture (2–6), but until now no satisfactory method has been developed.

As the fetus is genetically foreign to the maternal organism, one unique and important function of placenta may be to serve as an immunological barrier to maternal alloreactivity (7). One persistent suggestion is that trophoblast lacks HLA-A,B,C antigens, and both immunohistological and biochemical data exist supporting this suggestion (8–10). However, the presence of HLA-A,B,C antigens on early nonvillous trophoblasts has also been reported (11). Quantitative data on neither expression nor regulatory mechanisms of HLA-A,B,C antigens for individual placental trophoblasts or other cells is available.

In this paper, we establish a method for isolating specific placental cell populations and define the basic characteristics of human placental cells in suspension using coordinate two-color and light-scatter fluorescence-activated cell sorter (FACS)[1] analysis and sorting. We present data showing at least five populations of placental cells and describe their cell surface antigens, size, frequency, genetic origin, tissue-specific enzymes, and morphological appearances. The major population is clearly identified as cytotrophoblasts.

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[1] *Abbreviations used in this paper:* β2m, β_2-microglobulin; FACS, fluorescence-activated cell sorter; LAP, liver alkaline phosphatase; NCS, newborn calf serum; PAP, placental alkaline phosphatase; PBL, peripheral blood lymphocytes; PI, propidium iodide; TR, Texas red.

The apparent lack of HLA-A,B,C antigens and β_2-microglobulin (β2m) on placental cytotrophoblasts prompted us to analyze the underlying molecular mechanisms. The expression of HLA-A,B,C antigens on the cell surface could be controlled by the rate of transcription of the gene for either of the two chains or at any of a number of posttranscriptional steps, including mRNA processing, degradation, or translation. It is also possible that the HLA-A,B,C heavy chain and β2m proteins are synthesized normally and that some other cellular defect prevents the proper assembly and transport of the heterodimeric molecules to the cell surface. In order to distinguish among these possibilities, we used HLA-A,B,C and β2m cDNA probes in Northern blot hybridizations, and found that human cytotrophoblasts have extremely small amounts of HLA-A,B,C mRNA, but ~10% of the levels of β2m mRNA found in lymphoid cells.

Materials of Methods

Isolation of Placental Cells. Placentas were obtained immediately after uncomplicated deliveries or Caesarian sections at term. Chorionic membrane and umbilical cord were stripped, and the material side of the placenta was scraped with a scalpel to remove the bulk of decidual cells. Placentas were then minced with scissors and washed extensively with RPMI-1640 (Irvine Scientific, Santa Ana, CA) to reduce blood contamination. The crudely dissected placental villi were cut into pieces of about 1 mm^3, washed through gauze to remove contaminating blood cells, and then pelleted at ~300 *g* for 10 min. Pellets were resuspended in 0.5% trypsin, 0.2% EDTA solution at ~2.5 ml of solution for each gram of tissue, and agitated gently at 37°C for 30 min. Proteolysis was stopped by addition of 3–4 volumes of 5% newborn calf serum (NCS) in RPMI-1640, and centrifugation at 500 *g* for 10 min at room temperature. The clear supernatant fluid was discarded. The loose pellet was resuspended in an equal volume of 0.03% DNase in phosphate-buffered saline and digested at 37°C for 30 min in order to remove viscous DNA-like material associated with the tissue. Free single cells were separated from undigested tissue fragments by passage through a double layer of gauze. After the remaining erythrocytes were lysed with 0.165 M NH_4Cl solution on ice for 5 min, cells were washed, resuspended in 5% NCS RPMI-1640, and counted. Viability was determined by ethidium bromide-acridine orange staining (12), and was always >90%. Usually $1–2 \times 10^6$ cells/g wet tissue were recovered from full-term placentas. Before we adapted this method, various concentrations of collagenase and trypsin were used with yields, viability, and HLA antigen densities inferior to those of the method described.

Monoclonal Antibodies and Avidin-Texas Red for Immunofluorescence Staining. Monoclonal antibodies MB40.5 (13), W6/32 (14), which identify framework components of HLA-A,B,C heavy chain, PA2.1 (15), which reacts specifically with HLA-A2 antigen, and BBM.1 (16), which reacts with β2m whether in association with HLA-A,B,C heavy chain or not, were kindly provided by Dr. P. Parham, Stanford University. Monoclonal antibodies, anti-β2m (L368, which reacts when associated with HLA-A,B,C) (17), anti-HLA-DR (17), $^-$HLe1, a pan-leucocyte antibody (18), and $^-$LeuM1, a monocyte/macrophage lineage antibody (19) were provided by Becton-Dickinson Monoclonal Center, Mountain View, CA. Anti-Trop-1 and anti-Trop-2, which are monoclonal antibodies specific to human trophoblast, were previously produced in our laboratory (20). Titrations with both first- and second-step reagents were carried out on known target cells and saturating concentrations were used in this study. Biotin-coupled indirect fluorescence and direct fluorescence labeling procedures have been described (21). Texas red–labeled avidin was prepared as described previously (21). All reagents were stored at 4°C with 0.1% sodium azide and centrifuged immediately before use.

Immunofluorescence Staining. Single cell suspensions were stained in 50–150 μl total volume in microtiter wells. For two-color staining, 10^6 placental cells were incubated in a

mixture of a first antibody conjugated with fluorescein and a second antibody conjugated with biotin for 20 min on ice. After 2 washes, avidin conjugated with TR was added, incubated on ice for 20 min, and washed twice. Propidium iodide (PI) was added (50 μl per well at 1 μg/ml) to label dead cells (22). Staining medium was the same as that used for isolation of placental cells.

Two-color immunofluorescence analysis and sorting was carried out as described elsewhere (22). We always confirmed that a pair of antibodies or reagents did not inhibit the staining of each other. Briefly, several improved analytical techniques were used. Dead cells stained with PI were excluded by gating on the extremely high red fluorescence signal, since large dead cells cannot be excluded from fluorescence analysis simply by gating out of cells with low forward light scatter signals. For "off line" analysis, we collected correlated two fluorescent and forward light scatter measurements "list mode" on a VAX 11/780 computer on 20,000 PI-excluding (live) cells.

Y-Chromatin Detection. For fluorescent microscopic analysis, the cells in each subpopulation were sorted directly into wells on microscope slides and then centrifuged, fixed, and stained with quinacrine as described previously (23).

Specific identification of placental alkaline phosphatase (PAP) or liver alkaline phosphatase (LAP) used immunoprecipitation followed by an enzymatic assay (24). Briefly, cells are lysed, specific antibody is added, and the complex is precipitated with Staphylococcus A. Alkaline phosphatase activity in the supernatant is determined before and after precipitation of PAP or LAP. The difference represents the percentage of the specific isoenzyme in the total activity. Phosphatase activity is measured by release of *p*-nitrophenol (by spectrophotometry at 405 nm) from *p*-nitrophenyl phosphate at 2 mg/ml in monoethanolamine buffer (pH 11.2) at 37°C.

Morphological examination was carried out with smears prepared from sorted cells. Smears were fixed with methanol and stained with Giemsa.

Cell Lines. LCL-721, a human lymphoid cell line (25), which expresses HLA-A,B,C and β2m to a similar degree as normal peripheral lymphocytes; Daudi, a human Burkitt lymphoma cell line, which lacks surface HLA-A,B,C and β2m; and F9, a mouse undifferentiated teratocarcinoma cell line that lacks H-2 and β2m protein and mRNA (26).

cDNA Probes. An HLA-B7 cDNA clone that cross-hybridizes with HLA-A,B,C genes and mRNA and contains a 1400-bp insert (27) was generously provided by Dr. S. Weissman (Dept. Human Genetics, Yale Univ.). A human β2m cDNA clone containing 328 bp of protein coding sequence and 217 bp of 3′untranslated region (28) was the kind gift of Dr. K. Itakura (City of Hope Hospital, Duarte, CA). For both clones the largest Cfo I fragment (containing primarily cDNA insert plus 337 bp of pBR 322) was isolated and labeled with ^{32}P by nick translation to a specific activity of 2×10^8 cpm/μg.

RNA Gels and Hybridization. RNA was extracted from frozen cell pellets by homogenization in the presence of 4 M guanidine thiocyanate, centrifugation through a cushion of 5.7 M cesium chloride, and ethanol precipitation as described by Chirgwin et al. (29). The amount of RNA was determined by measuring the A260. 120 μg of total RNA was obtained from 4×10^7 sorted placental cytotrophoblast cells. 12 μg of each RNA sample in 50% formamide, 2.2 M formaldehyde, and 1 × MOPS buffer was denatured by heating for 5 min at 70°C, quick chilled, and electrophoresed in 1.5% agarose gels containing 2.2 M formaldehyde and 6.25 μg/ml ethidium bromide in 1 × MOPS buffer. RNA separated by electrophoresis was subsequently transferred to nitrocellulose filters according to the procedure of Thomas (30). Blots were hybridized to [^{32}P]-labeled HLA-A,B,C or β2m probes for 18 h at 42°C in 40% formamide, 4 × SSC, 1 × Denhardt's solution, 20 mM Tris pH 7.6, 0.1% SDS, 100 μg/ml denatured herring sperm DNA, and 50% dextran sulfate. The filters were then washed at room temperature with four changes of 2 × SSC and 0.1% SDS for 20 min each, followed by three washes at 55°C for 1 h each with 0.1 × SSC and 0.1% SDS. (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate). The filters were then dried and exposed to XAR-5 film (Kodak) at −70°C overnight. The intensities of the hybridizing HLA-A,B,C (2.0 kb) and β2m (0.95 kb) mRNA bands after autoradiography were quantitated by densitometry. The positions of the 18S and 28S ribosomal RNA markers were determined by the ethidium bromide staining pattern of the gel before blotting.

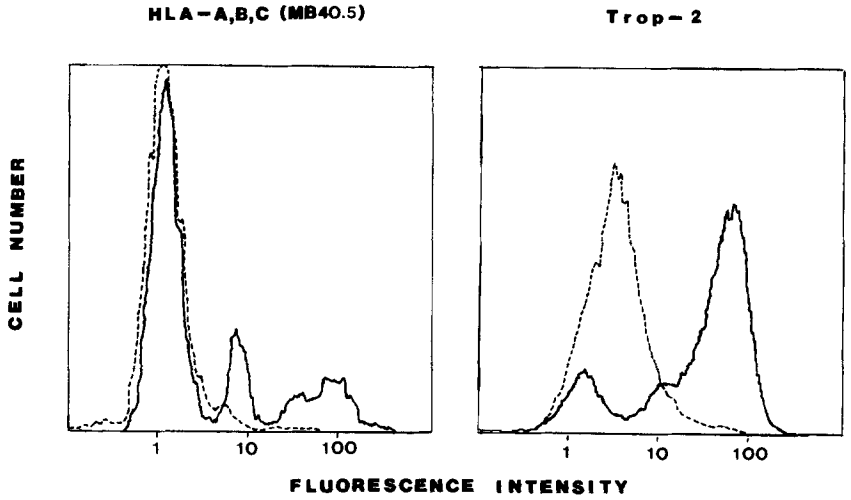

FIGURE 1. FACS fluorescence intensity histograms. Placental cells were stained with fluorescein-conjugated MB40.5, which detects a framework determinant on HLA-A,B,C heavy chain (left panel) or with biotinated anti-Trop-2, which reacts with human normal trophoblast and choriocarcinoma (right panel) followed by Texas-red-avidin. Note that fluorescence intensity is shown on a logarithmic scale.

Results

Single-color Analysis. Fig. 1 (left panel) shows fluorescence histograms (on a logarithmic scale) of placental cell suspensions stained with a fluorescein-conjugated anti-HLA-A,B,C framework determinant monoclonal antibody (MB40.5). We found three major populations on the basis of the relative amounts of HLA-A,B,C antigens on these cells. Interestingly, >70% of the cells have no detectable staining with this antibody. Roughly 10% have weak but definite staining and ~8% have bright staining equal to or greater than that found with normal peripheral blood lymphocytes (PBL).

To determine whether the cell isolation procedures used change the density of HLA-A,B,C antigens, the effect of the same enzymatic treatment on antigen density of normal PBL and SCH cells, an HLA-A,B,C antigen–positive choriocarcinoma cell line (31) was tested. We found no significant effect of this treatment on antigen density. The cell surface antigens detected by other monoclonal antibodies described below were also not significantly changed on appropriate target cells after this treatment.

Previously, we described two monoclonal antibodies (Trop-1 and Trop-2) specific for trophoblast antigens on choriocarcinoma cell lines and syncytio- and cytotrophoblast in placental sections (20). With these antibodies, we found only weak staining of a majority of suspended placental cells by anti-Trop-1 (Fig. 4), but a broad bimodal distribution of staining with anti-Trop-2 (Fig. 1, right panel).

Two-color Analysis. Fig. 2*a* depicts a representative staining pattern showing five distinct cell populations identified by differences in the correlated fluorescent staining intensities for HLA-A,B,C antigens and Trop-2 antigen on individual cells. We designated these five populations A, B, C, D, and E as shown in Fig. 2*a*. Simultaneous Trop-2 and HLA-A,B,C staining clearly resolves the HLA-

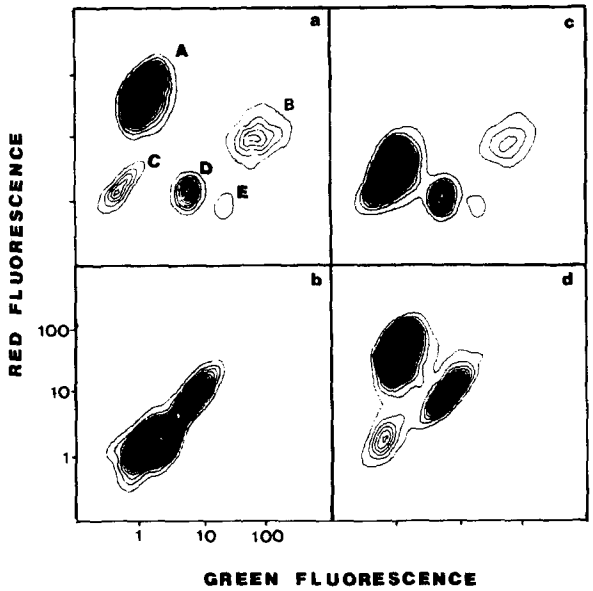

FIGURE 2. Representative contour plots of two-color immunofluorescence staining of placental cells. *a*, placental cells were stained with fluorescein-conjugated MB40.5 (green) and biotinated anti-Trop-2 followed by Texas-red-avidin (red); *b*, unstained (autofluorescence) profile; *c*, stained with MB40.5 alone; *d*, stained with anti-Trop-2 alone. Each contour represents a line connecting points of equal cell frequency. The contours were computer-generated from correlated fluorescein and Texas-red measurements on 20,000 cells (log-log contour displays).

A,B,C antigen–negative population seen in Fig. 1 into populations A and C. Population D, which is HLA-A,B,C weak but Trop-2 negative, is clearly resolved from both A and C. Population E is the bright HLA-A,B,C staining population in Fig. 1, which we see in Fig. 2,*a* and *c* to be Trop-2 negative. To help understand these results, we present in Fig. 2*b*, contour plots of red and green autofluorescence (fluorescence detected with no added stains) and in Fig. 2,*c* and *d*, contour plots of red and green fluorescence after staining for HLA-A,B,C or Trop-2 alone. Population B is HLA brightly positive and Trop-2 negative. Population B lies in an apparently Trop-2 positive area of the plot in Fig. 2*d*, but one notices that it lies in exactly the same area in Fig. 2*b* (autofluorescence) obtained with no fluorescent antibodies; thus it is really Trop-2 negative. In Fig. 2*c*, population B is seen to be spread out and moved to the right by staining with fluorescein-conjugated anti-HLA-A,B,C. In this pattern we also see two other small subsets of HLA-A,B,C–positive cells lacking Trop-2 antigen. Population C is a subset of cells negative for both reagents and appears at the origin.

We present in Table I the fraction of cells from eight normal-term placentas lying within each population. Population A is clearly the predominant cell population. The other four subpopulations occur with somewhat variable, but smaller frequencies.

Fig. 3 shows the size differences indicated by the forward angle light scatter profiles. These revealed that the HLA-A,B,C–negative and Trop-2-positive cells

TABLE I
*Cell Populations in Term Placenta**

	A	B	C	D	E
Cell size	Intermediate/large	Large	Small	Small	Small
Frequency	74%	8%	2%	6%	2%
(range)	(66–83)	(2–17)	(1–5)	(1–20)	(0–5)
Cell surface antigens					
Trop 1	+	–	–	–	–
Trop 2	++	–	–	–	–
HLA-A,B,C	–	+++	–	+	++
β2-microglobulin	–	+++	–	+	++
HLA-DR	–	–	–	–/+	+/–
HLe-1	–	–/+	–	+/–	+
LeuM-1	–	–	–	+/–	–
Cell origin					
Y-body in male placenta	43%	38%	ND	6%	1%
HLA-A2 cells [mother, A2(+); fetus, A2(–)]	–	0%	ND	<30%	<10%
Placental alkaline phosphatase activity	+	++	ND	+	ND

* 8 term placentas.

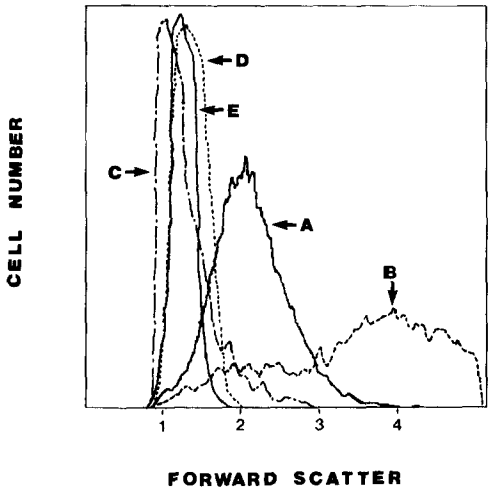

FIGURE 3. Forward light scatter profiles of the five placental cell populations in Fig. 2. Scatter curves present linear scale (abscissa) vs. relative cell number (ordinate). Dead cells were grated out from analyses by propidium iodide staining.

(A population) are relatively large as judged by light scatter measurement. In contrast, the C, D, and E cells are small and homogeneous in size. As shown in Fig. 3, the B population gives very high light scatter signals, which are confirmed to be due to very large cells by direct morphological examination of sorted B cells as described below under *Morphology*.

Staining with W6/32 and MB40.5 (anti-HLA-A,B,C framework antibodies) and two anti-β2m monoclonal antibodies, L368 and BBM.1, gave identical results (data not shown). In order to determine the amount of a series of other cell

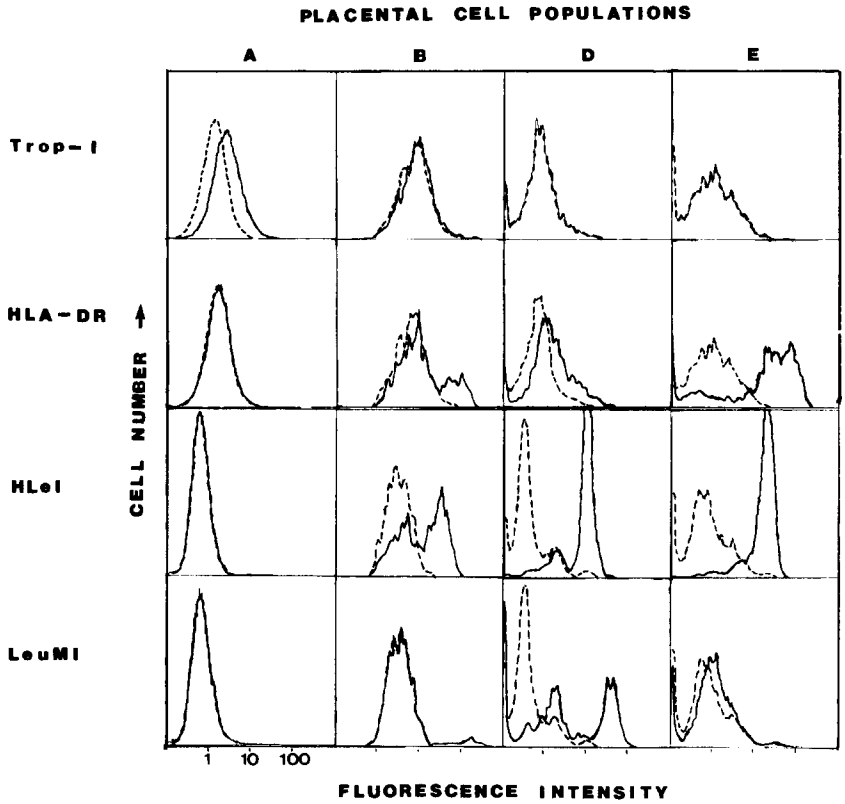

FIGURE 4. Immunofluorescence staining histograms of each placental cell population defined in Fig. 2. Placental cells were co-stained with fluorescein-conjugated MB40.5 and either biotinated anti-Trop-1 or anti-HLA-DR, or with biotinated W6/32 and either fluorescein-conjugated anti-HLe1 or anti-LeuM1 followed by Texas-red-avidin. Placental "A," "B," "D", and "E" populations were identified by correlated HLA-A,B,C fluorescence intensity and light scatter intensities. FACS curves present log fluorescence (abscissa) vs. relative cell number (ordinate). Solid lines show stained histograms, and dashed lines show red or green autofluorescence histograms of each population.

surface antigens on populations A, B, D, and E defined in Fig. 2*a*, we identified these populations by correlating HLA-A,B,C fluorescence and light scatter intensities. The HLA intensity distributions and light scatter distributions are shown in Figs. 1 and 3, respectively. We then used the computer-stored list mode data to determine the distributions of Trop-1, HLA-DR, HLe1, and LeuM1 on the A, B, D, and E populations by computer gating with windows defined by both HLA-A,B,C and scatter intensities. These distributions are shown in Fig. 4. Anti-Trop-1 weakly stains only population A, consistent with the other trophoblast-specific antibody Trop-2, shown in Fig. 2. HLA-DR is present on a small and variable fraction of population B and D cells, and on most E cells. HLe1 (a pan leukocyte antigen not found on fibroblasts [18]) is absent from population A but is on some B, most D, and most E cells. LeuM1 (present on mature granulocytes and monocytes [19]) is also absent from population A. Anti-LeuM1 stains only a small number of D cells. On the basis of these data with

TABLE II

Placental Alkaline Phosphatase in Placental Populations

Cells	Alkaline phosphatase activity (absorbance at 405 nm)			% PAP activity
	Supernatant	Anti-PAP	Anti-LAP	
Placenta populations				
Unfractionated	0.108	0.000	0.128	>96
A	0.048	0.000	0.047	>97
B	0.235	0.000	0.232	>98
D	0.073	0.017	0.074	>77
Lymphocyte*	0.000	0.000	0.000	0
BeWo‡	1.463	0.049	1.506	>97
LAP§	0.361	0.430	0.004	1

* Peripheral blood lymphocytes were isolated from freshly drawn venous blood by flotation on a Ficoll/Paque gradient.

‡ Gestational choriocarcinoma cell line.

§ Purified liver alkaline phosphatase.

Cell extract was reacted with specific antibody against placental (PAP) or liver alkaline phosphatase (LAP), and the enzyme-antibody complex precipitated. The supernatant was then assayed for enzyme activity by measuring the hydrolysis of *p*-nitrophenylphosphate. Activity remaining in the supernatant indicates the specific antibody did not react with isoenzyme, and absence of enzyme activity in the supernatant indicates that an isoenzyme did react with the specific antibody. The percentage of the total activity due to the liver or placental isoenzymes was calculated for each assay.

anti-HLA-DR, −HLe1 and −LeuM1, the B, D, and E populations probably contain contaminating blood cells. These data also provide internal controls for staining by these antibodies and confirm the absence of HLA-DR, HLe1, and LeuM1 on the cells of population A.

These cell surface phenotypes (summarized in Table I) strongly suggest that the A population is derived from trophoblast lineage cells, and rule out the possibility that this cell population belongs to monocyte/macrophage lineage cells.

Tissue-specific Alkaline Phosphatases of Sorted Cells. As shown in Table II, PAP (and not LAP) was clearly detectable in extracts from the A population, leading us to conclude it is of placental origin, since human placental-type alkaline phosphatase is generally believed to be a tissue-specific marker for placental tissue (32). Populations B and D also have PAP activity, suggesting that all or a part of these populations is of placental origin.

Fetal or Maternal Origin of Each Population. As human placenta is an intricate structure made of fetal and maternal components, careful identification of the genetic origin of dispersed cells is extremely important. To determine the origin of these cells, each sorted cell population prepared from male placentas was scored by fluorescence microscopy for Y-chromatin–containing cells after staining with quinacrine mustard. Data in Table I show 43% and 38% of cells in populations A and B, respectively have Y-chromatin, whereas much lower frequencies of D and E cells have Y-chromatin. Generally only 30–50% of male cells reveal Y-chromatin (33). Applying a correction based on this underenumer-

ation, we estimate that the A and B populations are almost exclusively of fetal origin, while the D and E populations contain cells of fetal and maternal origin.

In order to confirm the genetic origin of these cell populations by an independent approach, placental cells were obtained from a pregnancy in which the baby was typed as HLA-A2-negative on cord blood lymphocytes and the mother was typed HLA-A2-positive using her peripheral blood lymphocytes. Then, those placental cells were stained with fluoresceinated MB40.5 (anti-HLA-A,B,C antibody, monomorphic determinant) and biotinated PA2.1 (anti-HLA-A2 antibody, polymorphic determinant), which thus can distinguish maternally derived cells from fetally derived cells among HLA-A,B,C-positive populations. The absence of HLA-A2 antigen on B population cells from a placenta of an HLA-A2 positive mother is confirmation of the fetal origin of this population (data shown in Table I). In contrast, D and E populations from this placenta have a subset stained by PA2.1, indicating the presence of maternal HLA-A2 antigen not shared by the fetus; therefore these populations are further divisible into fetally and maternally derived subpopulations. These data are also clearly consistent with the low frequency of Y-chromatin in these populations obtained from male placentas.

Morphology. We initially recognized several morphologically distinct cell types in the placental cell suspensions following trypsin digestion. Cell smears were prepared from each sorted fraction. Representative cell types in smears from sorted populations A and B are illustrated in Fig. 5. Morphological examination showed that cell sizes in each population are consistent with the forward scatter data described above. The cells in population A were relatively homogeneous in appearance and contained large round or irregularly shaped nuclei with a thick or thin cytoplasm (Fig. 5, *a* and *b*). Most of the cells (1,521/1,526 = 99.7%) were uninucleate and the rest had two nuclei. We did not find any typical multinucleated extremely large syncytiotrophoblasts or blood cells. Plasma membranes were occasionally irregular.

The B population was somewhat heterogenous in morphology: the nuclear to cytoplasmic ratios were variable. Again, nearly all of the cells (1,301/1,323 = 98.3%) were uninucleate. It is important to note that large, typical Hofbauer cells, characterized by strongly vacuolated cytoplasms, were found in the B population (Fig. 5*c*) but not in the A population. Hofbauer cells are seen only in stromal areas of placental tissue sections (34). Some of the remaining cells in population B (see Fig. 5, *d* and *e*) are large cells (approximate cell size 25–80 μm) that may also be stromal cells. In the D population, we identified a number of polymorphonuclear cells and some other cells that we could not classify (not shown). In population E we saw only typical lymphocytes and various types of blood cells (not shown).

We summarize in Table I the antigenic phenotypes, genetic origins, tissue-specific phosphatase activities, and morphological properties of cells in the placental populations. These observations provide multiple criteria for identifying placental cell subpopulations in suspension. The majority A population consists of homogeneous, trophoblast lineage cells that our evidence indicates are cytotrophoblasts. The B cell population appears to consist mainly of stromal cells. It is entirely fetal in origin, Trop-1- and Trop-2-negative, consists mostly

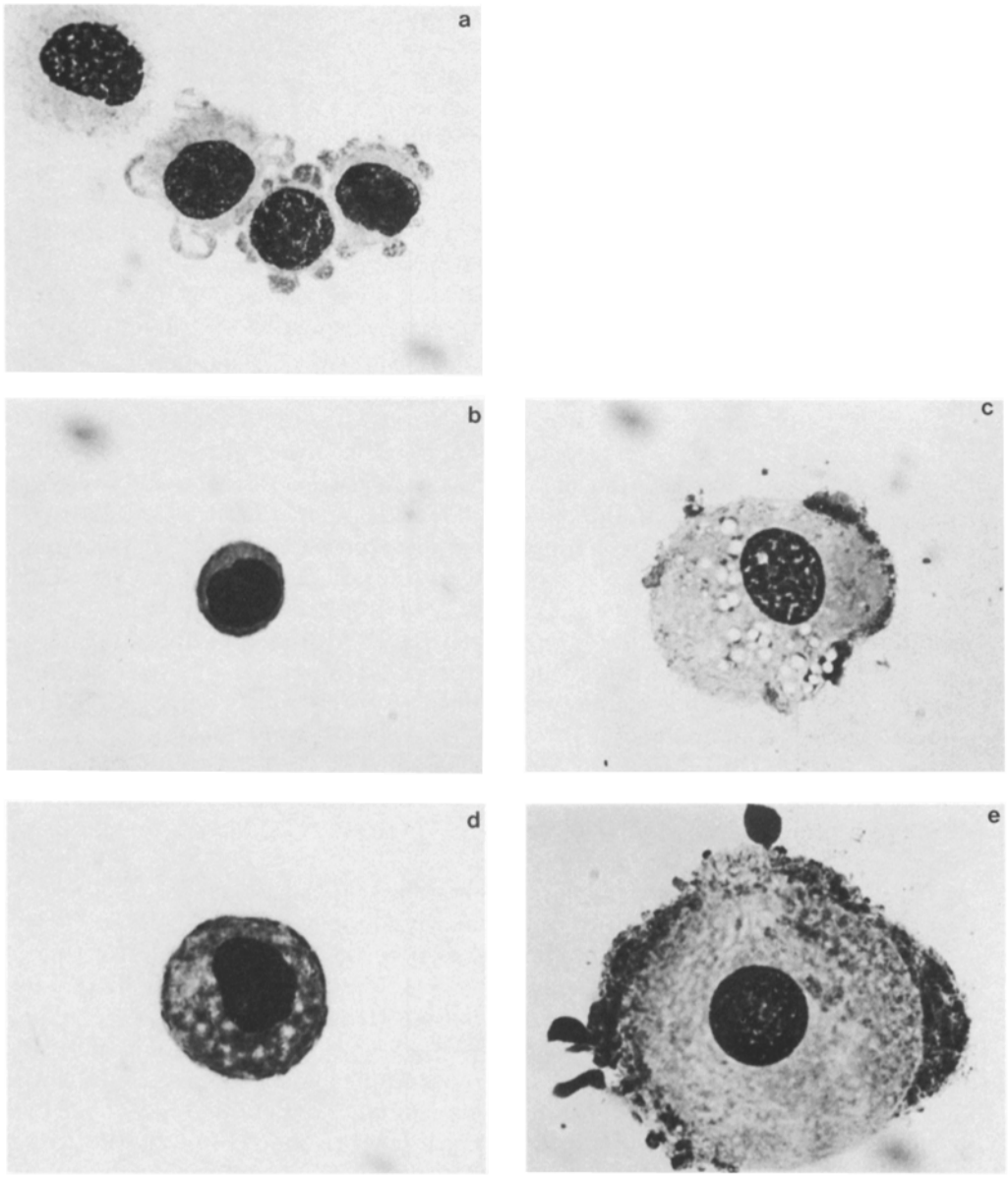

FIGURE 5. Representative photographs (stained with Giemsa) of cells from populations A and B. Panels *a* and *b* show cytotrophoblasts with irregular and smooth membranes, respectively, found in population A. Panels *c*, *d*, and *e* show a Hofbauer cell, a large and a giant stromal cell, respectively, found in population B. All photos were taken at the same magnification.

of very large cells including typical Hofbauer cells characteristic of stromal cells, and also contains some fetal origin cells that stain with HLe1. The latter cells are presumably fetal macrophage-like cells.

Populations C, D, and E contain a small proportion of the total placental cell suspensions (maximum of 10%). These populations have (contaminating) blood

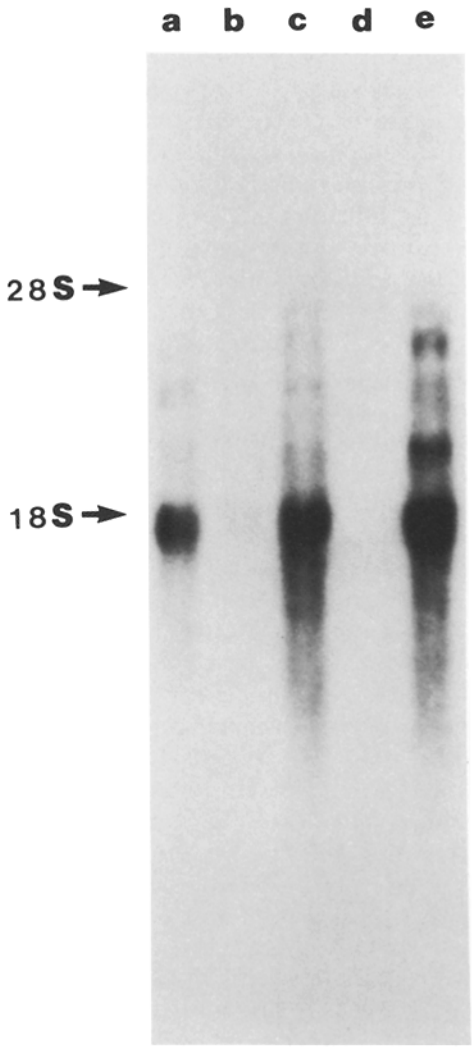

FIGURE 6. Autoradiography of Northern blot hybridization of human placental cytotrophoblast RNA with an HLA-A,B,C heavy chain cDNA probe. Total cellular RNA was isolated, subjected to agarose gel electrophoresis, blotted to a nitrocellulose filter, and hybridized with [^{32}P] labeled HLA-A,B,C cDNA probe as described in Materials and Methods. 12 μg of RNA was loaded in each lane. Lane *a*, whole placental cells; *b*, purified cytotrophoblasts; *c*, LCL-721 cells; *d*, F9 cells; *e*, Daudi cells. The migration position of the 28S and 18S ribosomal RNAs was identified by ethidium bromide staining.

cells, mixed genetic origins, and variable frequencies of reactivity with the different monoclonal antibodies used. In order to obtain pure cytotrophoblasts from dissociated placental cells, these small populations should be removed.

For the purpose of analyzing the underlying molecular mechanism accounting for the lack of HLA-A,B,C antigen on cytotrophoblasts, 4×10^7 cytotrophoblasts (population A) were sorted as described above and quickly frozen at −80°C. Viability after sorting procedures was >95%.

Total RNAs extracted from these purified cytotrophoblasts and various control cell lines were electrophoresed, blotted onto nitrocellulose filters, and hybridized with HLA-A,B,C or β2m cDNA probes to obtain information about both the sizes and the relative amounts of the corresponding mRNA species. The original placental cell suspensions before sorting, LCL-721 (lymphoblastoid) and Daudi (Burkitt lymphoma) cell lines, were used as controls. We also used the mouse F9 cell line as a negative control, since it lacks H-2 and β2m mRNAs (26), and therefore should not hybridize with the human probes.

In Fig. 6, the major hybridizing bands (2.0 kb) in lanes *a*, *c*, and *e* represent mature HLA mRNA in RNA from unfractionated placental cells, LCL-721 cells and Daudi cells, respectively. The fainter larger species presumably represent nuclear precursors (unspliced or partially processed). F9 cells (lane *d*) showed no evidence for specific mRNA hybridization even after prolonged exposure. We found very faint 2.0-kb bands in RNA extracted from purified cytotrophoblast cells (lane *b*). The relative concentration of HLA-A,B,C mRNA were quantitated by scanning the autoradiogram with a densitometer. By comparison of the intensities of the bands obtained with the same amounts of RNA prepared from the purified cytotrophoblast fraction or LCL-721, the former contained ~300 times less HLA-A,B,C specific mRNA than the latter. We have shown that LCL-721 cells have about the same amount (1.05 times) of HLA-A,B,C mRNA as normal peripheral blood lymphocytes (footnote 2). These results indicate that placental cytotrophoblasts have considerably <1% of the level of HLA-A,B,C-specific mRNA (both mature and precursors) present in LCL-721 or normal peripheral lymphocytes.

In contrast, HLA-A,B,C mRNA is present in whole placental populations (Fig. 6, lane *a*) at ~40% the level of LCL-721 cells. This agrees well with our immunofluorescence data described above. Fig. 6 also shows that Daudi cells, which like the cytotrophoblasts lack surface expression of HLA-A,B,C antigens, contain high levels of HLA-specific mRNA (lane *e*) as recently shown (reference 35, footnote 1).

The β_2-microglobulin mRNA in purified placental cytotrophoblasts was analyzed in a similar manner (Fig. 7). The Northern blot shows that the labeled human β2m cDNA probe hybridizes to a 0.95-kb mRNA in both cytotrophoblasts (lane *b*) and LCL-721 cells (lane *c*), although the intensity of the band in the cytotrophoblasts was ~11% that in LCL-721 cells. Like the cytotrophoblasts, the Daudi cells also have clearly detectable levels of β2m mRNA (lane *e*), despite the lack of cell surface expression. As expected, the β2m probe did not hybridize to any mRNA inF9 cells (lane *d*).

Discussion

We have developed a method to identify and separate cell populations from human placental cell suspensions using immunofluorescence staining, FACS analysis, and sorting. The heterogeneity of suspended placental cells with respect to size, amounts of HLA-A,B,C and Trop antigens, autofluorescence, genetic

[2] Kawata, M., K. Sizer, S. Sekiya, J. R. Parnes, and L. A. Herzenberg. 1984. Differential expression of HLA-A,B,C antigens on choriocarcinoma cell lines: limited by mRNA for HLA heavy chain, not β2-microglobulin. Submitted for publication.

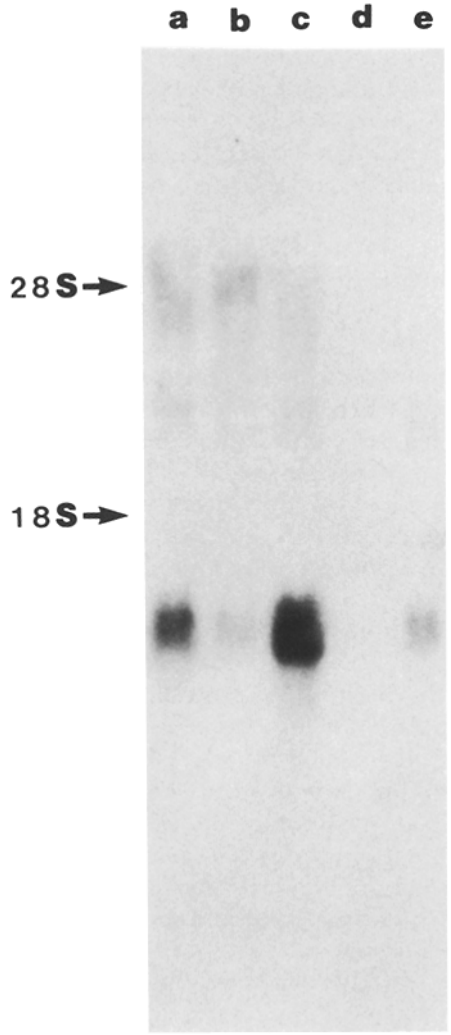

FIGURE 7. Autoradiography of Northern blot hybridization of human placental cytotrophoblast RNA with a β2-microglobulin cDNA probe. Procedures were identical to those described in Fig. 1. Lane *a*, whole placental cells; *b*, purified cytotrophoblasts; *c*, LCL-721 cells; *d*, F9 cells; *e*, Daudi cells.

origin, and the presence of blood cells and dead cells has previously hampered the preparation of pure subpopulations of placental cells. However, the addition of two-color correlated fluorescence, log amplification, and PI staining methods (22) to previous FACS methodology (36) is particularly helpful for resolving and sorting such mixed cell populations. We found that the most abundant cell population, A (66–83% of placental cell suspension), consists of fairly pure cytotrophoblasts. These cells express the cell surface antigens Trop-1 and Trop-2, are HLA-DR-, HLe1-, and LeuM1-negative, PAP activity–positive, Y-chromatin–positive (using male placentas), and are large mononuclear cells. Furthermore, we have obtained phenotypically similar cells from suspensions of chorionic

membrane that contains a layer of cytotrophoblasts (M. K., unpublished observation). This supports the identification of placental A cells as cytotrophoblasts. In confirmation of studies by others (8–10), we have shown that cytotrophoblasts at term have undetectable levels of HLA-A,B,C antigens on their surface.

Mammalian reproduction occurs under unique biological circumstances in which allogeneic and semiallogeneic cells take part in the processes of fertilization, implantation, and development of the fetus *in utero*. Although it has been suggested that a number of different mechanisms are involved in the protection of the fetus from a maternal alloreactive immune response (7), the absence of HLA-A,B,C antigens could certainly act as a fundamental barrier against maternal transplantation immunity.

A variety of explanations have been postulated to explain the absence of detectable transplantation antigens on trophoblast cells: (*a*) enzymatic removal of the determinants during cell preparation; this is strongly argued against by our findings that SCH line cells and normal blood lymphocytes show no loss of HLA-A,B,C antigens by identical treatments and that other placental cells obtained by our procedure retain HLA-A,B,C antigens. Of course we cannot rule out a slight loss of HLA antigens by preferential sensitivity of these antigens on cytotrophoblasts to trypsin or enzymes released by placenta during trypsinization; (*b*) masking of the antigenic determinants in some way by overlying membrane-bound mucoprotein or other molecules (37); (*c*) immunological masking of the determinants by preformed maternal alloantibody (this appears unlikely because we have been unable to detect human immunoglobulins on the surface of the cytotrophoblast population using double staining with anti-HLA-A,B,C and anti-human immunoglobulins, unpublished data); (*d*) lack of transcription of HLA-A,B,C and/or β_2-microglobulin genes; (*e*) decreased HLA-A,B,C and/or β2m mRNA stability; (*f*) posttranscriptional processing deficiencies for HLA-A,B,C and/or β_2-microglobulin mRNAs; (*g*) defects in the assembly of the heterodimer, its transport to or insertion into the plasma membrane.

To better define the molecular mechanism for the lack of trophoblast HLA-A,B,C antigen expression, we have examined the levels of mRNA corresponding to HLA antigens and β_2-microglobulin, respectively, in a purified population of cytotrophoblast cells. Using cDNA hybridization probes and Northern blot analysis, we found extremely low levels of HLA-A,B,C mRNA in the purified cytotrophoblast cells (300-fold lower than in LCL-721 or normal lymphocytes). These results are consistent with regulation at the level of transcription or, conceivably, stability of HLA-A,B,C mRNA.

In contrast to the results for the HLA-A,B,C mRNA, we found the level of β_2-microglobulin mRNA in cytotrophoblasts to be only ninefold lower than in the lymphoid cell line LCL-721. These results imply that β2m mRNA is not limiting the expression of surface HLA-A,B,C molecules in these cells, and that β2m and HLA-A,B,C synthesis are differentially regulated. Together with our recent experiments[2] showing that the amounts of HLA-A,B,C antigens on the surface of choriocarcinoma cell lines are proportional to the amounts of HLA-A,B,C mRNA and not to the amount of β2m mRNA, the present data strongly support the idea that the amount of HLA-A,B,C anitgens on the trophoblast cell surface is determined by HLA-A,B,C heavy chain mRNA levels, in contrast to

the case with Daudi where β2m translation limits the amount of surface HLA expression (39–41).

HLA-A,B,C antigens are expressed on almost all normal tissues (42). The results reported here are of biological interest because they demonstrate a tissue-specific limitation of HLA-A,B,C gene expression in a normal cell population, as well as a differential regulation of mRNA levels for HLA-A,B,C antigens and β_2-microglobulin.

Goodfellow et al. (9) reported that β2m could be found in the cytosol fraction of syncytiotrophoblast membrane preparations without significant amounts of HLA-A,B,C antigens. Syncytiotrophoblast cells are generally recognized to be differentiated derivatives of cytotrophoblasts (38), and in direct contact with the maternal circulation. Based upon these results and our current findings, it seems likely that the absence of HLA-A,B,C antigens on syncytiotrophoblasts is also controlled by the level of HLA-A,B,C mRNA and not by the level of β2m mRNA.

It is of considerable importance to follow the changes in the regulation of HLA and other alloantigenic molecules in the early stages of placenta and extraembryonic membrane development. Using mouse teratocarcinoma stem cell lines, a widely used model system for studies of normal early embryogenesis, one of us (43) and others (44) reported that H-2 and β2m mRNAs in undifferentiated teratocarcinoma cell lines are absent or appear 100 times less than in differentiated teratocarcinoma cells, and that both mRNAs appeared simultaneously along with differentiation induced by retinoic acid. Trophoblastic cells differentiate early in development as the first specialized cell lineage from a normal embryonal cell mass, and continuously localize between maternal tissue and the developing fetus. Although virtually nothing is known about the molecular events in early human embryogenesis, selective transcriptional repression of HLA-A,B,C heavy chain molecules, which are involved in the specificity for recognition of "self" and "not self," might be a unique regulatory system for trophoblastic lineage cells. Recent findings indicate that the DNA of the HLA-A,B,C genes of at least one trophoblastic cell line can not transfect for expression in a DNA mediated gene transfer system into mouse fibroblasts.[3] This is in sharp contrast with DNA from any other cell or tissue source examined.[4]

It is well established that antibodies directed against paternally inherited fetal HLA-A,B,C antigens are found in the sera of multiparous women (45), and that these antibodies are also elutable from full-term placentas (46). These observations suggest that the placenta acts not only as a potential source of alloantigenic stimulation, but also as an immunoabsorbent with alloantigenic specificity. The differential expression of HLA-A,B,C antigens on placental A and B cells, as shown in this study, suggests that placental cells expressing paternally derived HLA-A,B,C antigens are B population, stromal cells. It agrees with the finding of HLA-A,B,C on stroma in immunohistological studies by Faulk et al. (8). Thus, B cells as well as circulating fetal cells detectable by anti-paternal HLA antigens

[3] Alberti, S., and L. A. Herzenberg. Unpublished material.

[4] Hsu, C., P. Kavathas, and L. A. Herzenberg. 1984. Expression of cell-surface differentiation antigens on mouse L cells transfected with whole genomic DNA from non-expressing as well as expressing cells or tissues. Submitted for publication.

(47) may be a source of alloantigen responsible for inducing alloantibodies during pregnancy. The stromal cells are also good candidates for absorbers of anti-HLA antibodies known to be present in placenta.

We carefully examined placental cell suspensions for multinucleated cells and did not observe any. This is consistent with observations by Carr (48) that syncytial trophoblasts are broken readily by trypsinization. As cytotrophoblasts generally are recognized to be the precursors of syncytiotrophoblasts, it would be interesting to examine their formation from cytotrophoblasts in vitro. The availability of pure viable cytotrophoblasts provides a good starting point for such studies and also facilitates immunological, endocrinological, and molecular studies of human trophoblasts. This method may also provide the means to identify and collect cytotrophoblasts by transcervical chorion biopsy or possibly in maternal peripheral blood (49, 50) for direct biochemical and molecular diagnoses at early prenatal stages.

Summary

Human placental cell suspensions prepared by trypsin digestion were analyzed with several monoclonal antibodies on a multiparameter fluorescence-activated cell sorter (FACS). Five distinct cell populations were isolated on the basis of size and quantitative differences in the coordinate expression of cell surface antigens detected by monoclonal antibodies against an HLA-A,B,C monomorphic determinant (MB40.5) and against human trophoblasts (anti-Trop-2). By FACS analysis and after sorting we clearly identified the major cell population as cytotrophoblasts based on several independent criteria, including presence of trophoblast-specific surface antigens, Trop-1, and Trop-2; absence of all HLA class I, class II, and β_2-microglobulin (β2m) antigens; absence of the pan-leucocyte and monocyte antigens, HLe1 and LeuM1, respectively; presence of Y-chromatin in a male placenta; presence of placental and not liver alkaline phosphatase; and a large, mononuclear morphology. These procedures provide a reproducible method for obtaining highly purified human cytotrophoblast populations for further studies.

We measured by molecular hybridization (RNA or Northern blots) the HLA-A,B,C and β2m mRNA in total RNA extracted from sorted cytotrophoblasts. We find that normal human cytotrophoblasts have extremely small amounts of HLA-A,B,C mRNA: ~300 times less than that in the lymphoid cell line LCL-721 or normal lymphocytes. In contrast, they have ~11% the level of β2m mRNA present in LCL-721 cells. Thus, HLA-A,B,C antigen expression on human cytotrophoblasts is limited by the level of HLA heavy chain mRNA.

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