Purification, Characterization, and *in Vitro* Differentiation of Cytotrophoblasts from Human Term Placentae*

HARVEY J. KLIMAN*, JOHN E. NESTLER†, EDUARDO SERMASI, JEAN M. SANGER, AND JEROME F. STRAUSS III

Departments of Pathology and Laboratory Medicine (H.J.K., J.F.S.), Medicine (J.E.N.), Obstetrics and Gynecology (E.S., J.F.S.), and Anatomy (J.M.S.), University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

ABSTRACT. Highly purified functional cytotrophoblasts have been prepared from human term placentae by adding a Percoll gradient centrifugation step to a standard trypsin-DNase dispersion method. The isolated mononuclear trophoblasts averaged 10 μm in diameter, with occasional cells measuring up to 20–30 μm. Viability was greater than 90%. Transmission electron microscopy revealed that the cells had fine structural features typical of trophoblasts. In contrast to syncytial trophoblasts of intact term placentae, these cells did not stain for hCG, human placental lactogen, pregnancy-specific β_1-glycoprotein or low mol wt cytokeratins by immunoperoxidase methods. Endothelial cells, fibroblasts, or macrophages did not contaminate the purified cytotrophoblasts, as evidenced by the lack of immunoperoxidase staining with antibodies against vimentin or α_1-antichymotrypsin. The cells produced progesterone (1 ng/10^6 cells·4 h), and progesterone synthesis was stimulated up to 8-fold in the presence of 25-hydroxycholesterol (20 μg/ml). They also produced estrogens (1360 pg/10^6 cells·4 h) when supplied with androstenedione (1 ng/ml) as a precursor.

When placed in culture, the cytotrophoblasts consistently formed aggregates, which subsequently transformed into syncytia within 24–48 h after plating. Time lapse cinematography revealed that this process occurred by cell fusion. The presumptive syncytial groups were proven to be true syncytia by microinjection of fluorescently labeled α-actinin, which diffused completely throughout the syncytial cytoplasm within 30 min. Immunoperoxidase staining of cultured trophoblasts between 3.5 and 72 h after plating revealed a progressive increase in cytoplasmic pregnancy-specific β_1-glycoprotein, hCG, and human placental lactogen concomitant with increasing numbers of aggregates and syncytia. At all time points examined, occasional single cells positive for these markers were identified. RIA of the spent culture media for hCG revealed a significant increase in secreted hCG, paralleling the increase in hCG-positive cells and syncytia identified by immunoperoxidase methods. We conclude that human cytotrophoblasts differentiate in culture and fuse to form functional syncytiotrophoblasts. (*Endocrinology* **118:** 1567–1582, 1986)

THE human syncytial trophoblast is known to serve several roles in pregnancy. It mediates the transport of nutrients and immunoglobulins from the maternal to the fetal circulation and also functions as an endocrine organ, secreting steroid and protein hormones (1). However, the process by which the syncytial trophoblast is formed is a subject of controversy. While a number of researchers hold that mononuclear cytotrophoblasts form the syncytium through a process of differentiation and cell fusion (2–5), others have proposed that syncytial trophoblasts are derived through endomitosis (nuclear

division without cytokenesis) of cytotrophoblasts (6–8). The approach to these important questions regarding the formation of the syncytial trophoblast and control of its function could be facilitated by a reproducible system with which these events could be studied *in vitro*. While a number of investigators have examined placental explants and preparations of dispersed placentae in culture (9–12), a facile system in which highly purified cellular components are employed has not yet been described.

In the present report we characterize purified cytotrophoblasts isolated from term placentae by enzymic digestion and Percoll gradient centrifugation. The *in vitro* transformation of these mononuclear trophoblasts into functional syncytial trophoblasts through cell fusion has been observed.

Materials and Methods

Preparation of dispersed mononuclear trophoblasts

Initial processing of human term placentae was performed essentially as described by Hall *et al.* (13), except for the
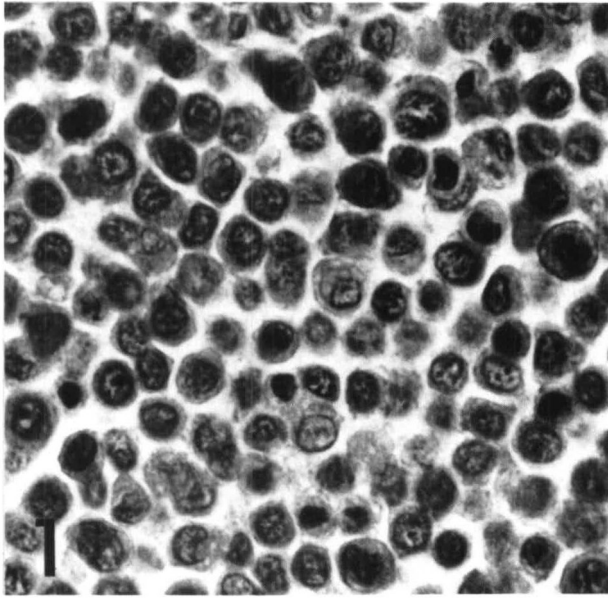
FIG. 1. Cells from middle region of Percoll gradient (density, 1.048–1.062 g/ml). A sample of the middle band was pelleted, fixed, and stained as described in *Materials and Methods*. Magnification, ×715.

addition of a Percoll (14) gradient centrifugation step. Normal term (36–42 weeks gestation) placentae were obtained immediately after spontaneous vaginal delivery or uncomplicated cesarean section. Several cotyledons were removed from underlying fibrous elements and rinsed thoroughly in 0.9% NaCl at room temperature. Soft villous material from the maternal surface was cut away from connective tissue and vessels until approximately 30 g were collected. The tissue was coarsely minced, transferred to 150 ml warmed calcium- and magnesium-free Hanks' solution (CMF Hanks') containing 25 mM HEPES, 0.125% trypsin (Sigma Chemical Co., St. Louis, MO), and 0.2 mg/ml DNase I (Sigma; 1,500 Kunitz units/mg), pH 7.4, and then incubated in a shaking water bath at 37 C for 30 min. The flask was set at an angle, and tissue fragments were allowed to settle for 1 min. Six aliquots (13.5 ml each) of the supernatant were layered over 1.5 ml calf serum (Grand Island Biological Co., Grand Island, NY) in 15-ml polystyrene conical

centrifuge tubes and centrifuged at 1000 × g for 5 min at room temperature. The resultant pellets were resuspended in 6 ml Dulbecco's Modified Eagle's Medium containing 25 mM HEPES and 25 mM glucose (DMEM-H-G) at room temperature. The remaining placental tissue was subjected to the digestion procedure two more times with the addition of 100 and 75 ml, respectively, warmed fresh trypsin-DNase solution. If, after any of the digestions, the supernatant was viscous, 10 mg DNase were added, and the mixture was allowed to incubate for an additional 10 min. The three resultant cell suspensions were pooled, centrifuged at 1000 × g for 10 min, and resuspended in 4 ml DMEM-H-G. This suspension was layered over a preformed Percoll gradient made up in Hanks' solution. The gradient was made from 70% to 5% Percoll (vol/vol) in 5% steps of 3 ml each by dilutions of 90% Percoll (9 parts Percoll:1 part 10X Hanks') with CMF Hanks' and layered in a 50-ml conical polystyrene centrifuge tube. The gradient was centrifuged at 1200 × g at room temperature for 20 min. After centrifugation, three regions were identified: bottom, containing red blood cells and occasional polymorphonuclear leukocytes; top, containing connective tissue elements, small vessels, and villous fragments; and middle, containing a relatively uniform population of mononuclear cells. The middle layer was removed, washed once with DMEM-H-G, and then resuspended in medium for tissue culture or fixatives for light or electron microscopy. The density at which the mononuclear cells banded in the gradient was determined by running parallel gradients loaded with density marker beads (Pharmacia, Piscataway, NJ).

Short term incubations of freshly isolated trophoblasts

Percoll gradient-purified trophoblasts (10^5–10^6 cells/ml) were incubated in medium consisting of DMEM containing 4 mM glutamine and 50 µg/ml gentamicin. In some experiments, the following were added to the medium either alone or in combination (in concentrations as indicated in the figure legends): androstenedione (Steraloids, Wilton, NH), 25-hydroxycholesterol (5-cholesten-3,25-diol; Steraloids), and aminoglutethimide (Ciba-Geigy, Summit, NJ). Incubations were carried out in a shaking water bath at 37 C for up to 4 h and were

TABLE 1. Immunocytochemical staining of intact placentae and isolated cytotrophoblasts

Antiserum	Intact placenta				Percoll gradient-purified cells
	Syncytiotrophoblasts	Cytotrophoblasts	Endothelial cells	Tissue macrophages	
Anti-hCG	±	—	—	—	—[a]
Anti-hPL	+	—	—	—	—
Anti-SP$_1$	+	—	—	—	—
Anticytokeratin	+	—	—	—	—
Antivimentin[b]	—	—	+	—	—
Anti-ACT[b]	—	—	—	+	—

Bouin's fixed intact placentae or cell pellets from the Percoll gradient (density, 1.048–1.062) were incubated with indicated antisera, as described in *Materials and Methods*. Immunocytochemical staining of various placental cell types and the cell pellet were graded as: no staining (—), variable staining (±), or strong staining (+).

[a] Rare single cells exhibited staining (see text).
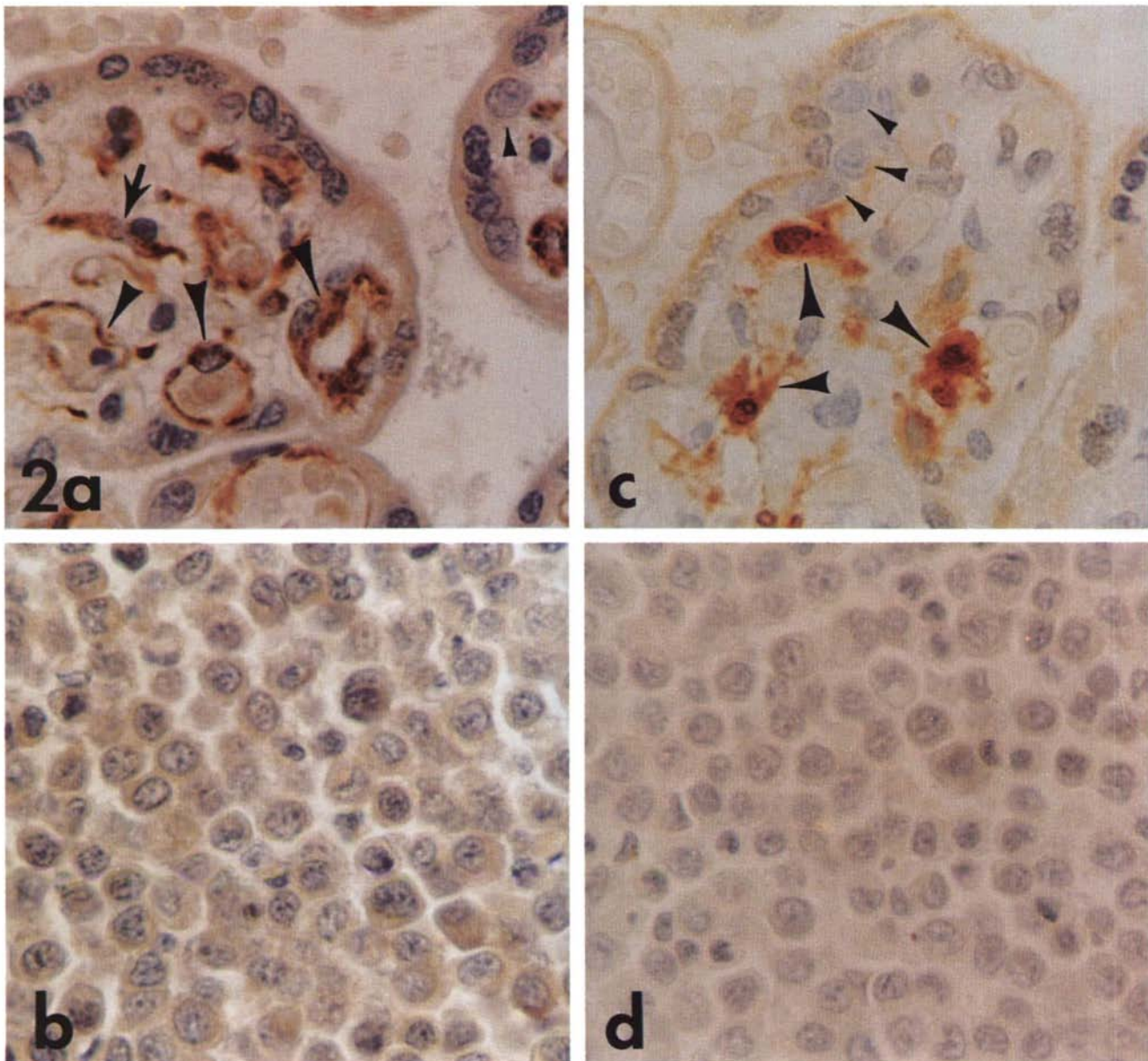[b] See Fig. 2.

FIG. 2. Immunoperoxidase staining for vimentin (a and b) and ACT (c and d) in intact term placenta (a and c) and Percoll gradient-purified cells (b and d). a) Antivimentin stains the endothelial cells of capillaries (*large arrowheads*) and fibroblasts (*arrow*), but not the syncytiotrophoblasts or cytotrophoblasts (*small arrowhead*). b) Percoll gradient-purified cells do not stain for vimentin. c) Anti-ACT stains the macrophages (Hofbauer cells) of the villous core (*large arrowheads*), but not the syncytiotrophoblasts or cytotrophoblasts (*small arrowheads*). d) Percoll gradient-purified cells do not stain for anti-ACT. All micrographs, ×750.

terminated by rapid freezing in an acetone-dry ice bath. Cells and media were analyzed for steroid contents by RIA.

Culture of trophoblasts

Percoll gradient-purified trophoblasts were diluted to a concentration of 8×10^5 cells/ml with DMEM-H-G containing 4 mM glutamine, 50 μg/ml gentamicin, and 20% (vol/vol) calf serum. The cells were plated into six-well (35-mm) Nunclon multidishes (Nunc, Roskilde, Denmark) with or without 22-mm square glass coverslips (no. 1) and incubated in humidified 5% CO_2-95% air at 37 C. At designated times, the culture media were removed and frozen at −20 C and replaced with fresh medium. At designated times, coverslips were removed for histological or immunocytochemical staining. Cells were cultured for up to 5 days.

Histology and immmunohistochemical staining of histological sections

Before processing the placentae for preparation of dispersed trophoblasts, several 2-mm pieces of the intact placentae were collected and fixed in Bouin's solution. After overnight fixation and paraffin embedding, serial sections at 5-μm intervals were prepared. Some sections were stained with hematoxylin and eosin, while other sections were placed on glass slides (coated
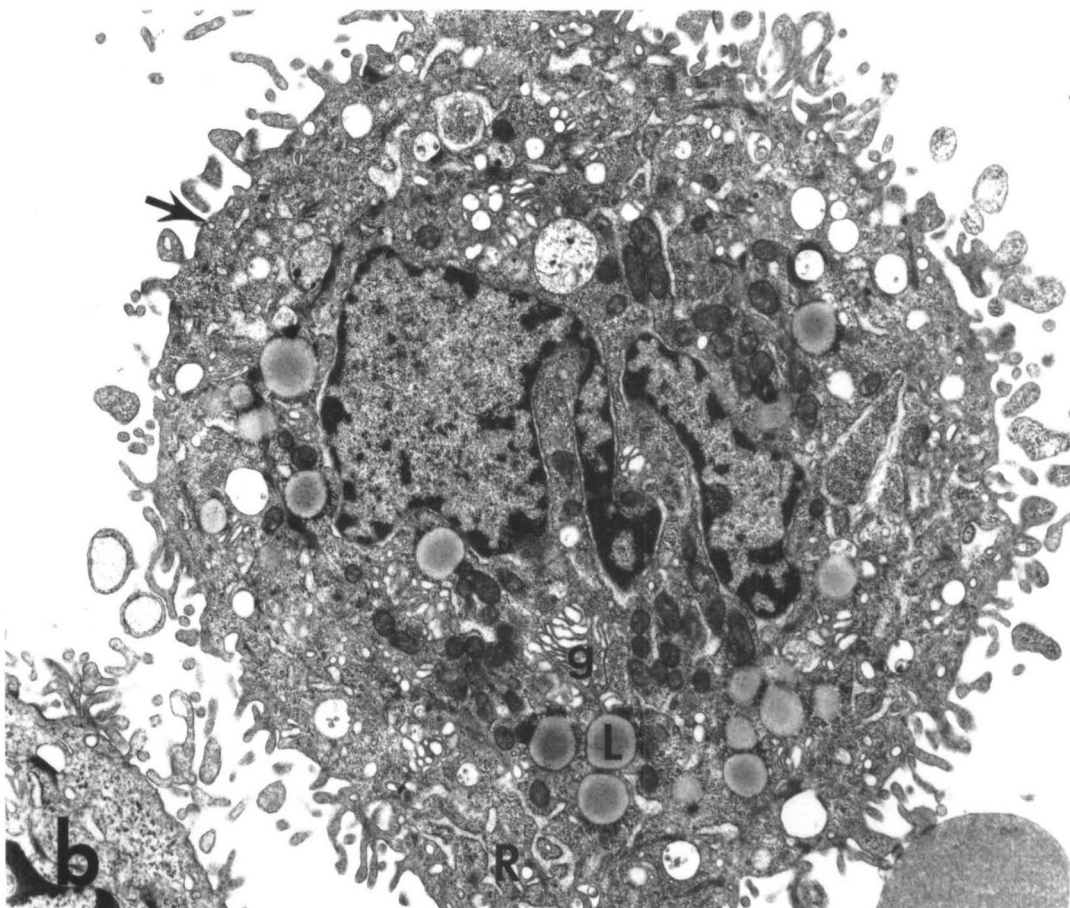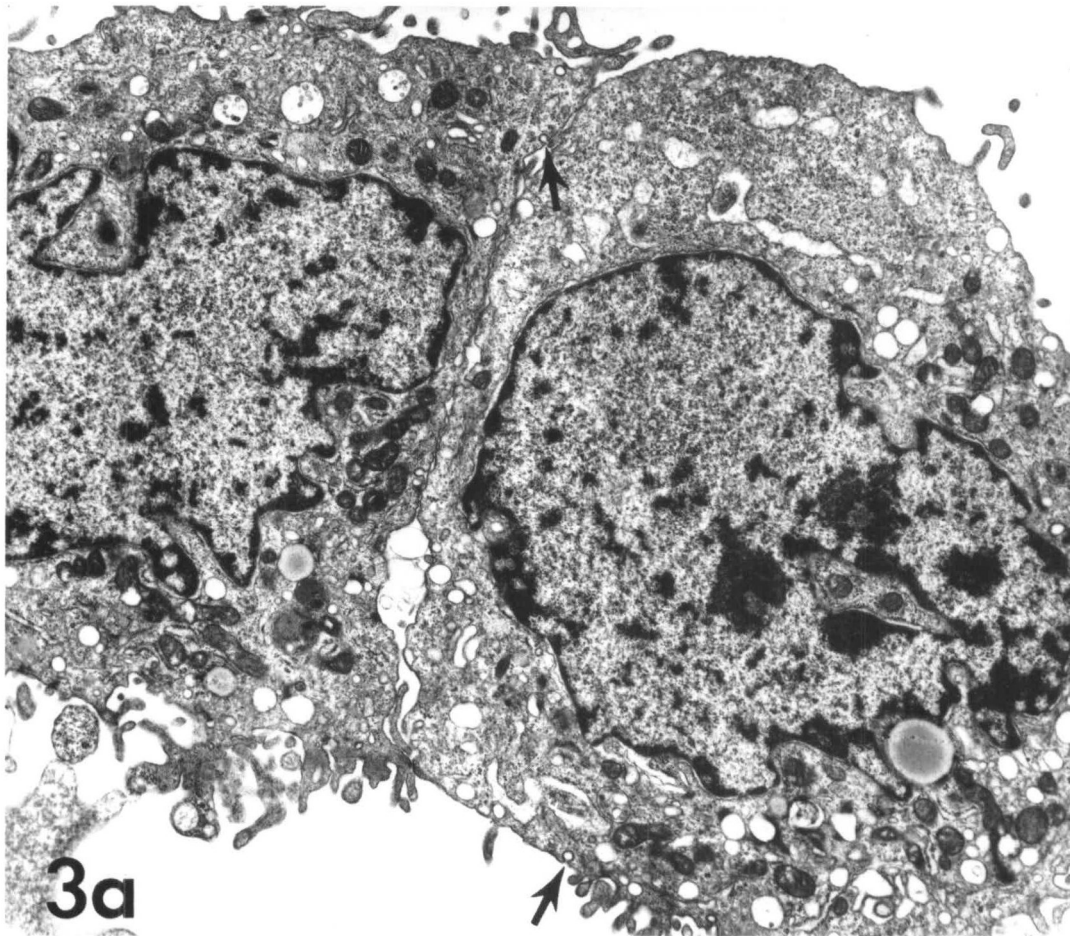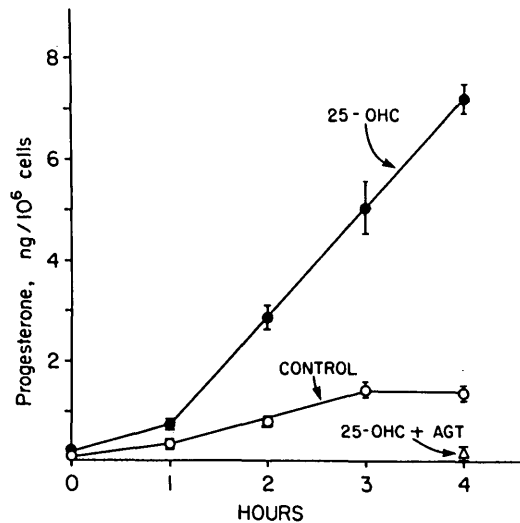
FIG. 4. Effect of 25-hydroxycholesterol (25-OHC) on progesterone synthesis by purified cytotrophoblasts. The cells were incubated at 37 C in medium alone (O), medium with 20 μg/ml 25-hydroxycholesterol (●), or medium with 20 μg/ml 25-hydroxycholesterol and 100 μM aminoglutethimide (AGT; Δ). Incubations were terminated at the indicated times by quick freezing, and progesterone contents of cells and media were determined by RIA. Values are the means of triplicate incubations at each time point.

TABLE 2. Estrogen synthesis by isolated cytotrophoblasts

Androstenedione (ng/ml)	Estrogen synthesis (pg/10^6 cells·4 h)
0	60 ± 38
0.1	440 ± 59
1.0	1363 ± 307

Percoll gradient-purified cytotrophoblasts were incubated for 4 h at 37 C in medium with the indicated amounts of androstenedione added. Cells and media were then quick frozen and thawed, and total estrogen content was assayed by RIA. Net estrogen synthesis was calculated by subtracting the estrogen content of cells and media at zero time. Values represent the mean ± SE of triplicate incubations.

with a film of 10% Elmer's white glue), dried at temperatures no greater than 60 C, and stored at room temperature until used (up to several months). Cell pellets of the Percoll-purified mononuclear cells were fixed and processed in a similar fashion. All subsequent steps were performed at 24 C. Paraffin was removed from the sections with xylene and rehydrated with graded ethanols, and then the slides were rinsed twice with 10 mM PBS, pH 7.4, and once with PBS-1% BSA (PBS-BSA). Endogenous peroxidase activity was quenched with a 15-min incubation with 0.6% hydrogen peroxide in absolute methanol. Nonspecific immunoglobulin-binding sites were blocked by a 30-min preincubation with 5% goat serum (Dako, Santa Barbara, CA) diluted in PBS. After this and all subsequent incubations, the slides were washed twice with PBS and then once

with PBS-BSA. Primary antisera raised in rabbits were diluted in PBS and applied for 45 min. These included antibodies against hCG (Dako) at a 1:1500 dilution, human placental lactogen (hPL; Calbiochem-Behring, La Jolla, CA) at 1:1000; pregnancy-specific β_1-glycoprotein (SP$_1$; Boehringer-Manheim, Indianapolis, IN) at 1:500, vimentin (Labsystems, Helsinki, Finland) at 1:600, and α_1-antichymotrypsin (ACT; Dako) at 1:1500. A monoclonal antibody against low mol wt cytokeratins (Labsystems, Chicago, IL) was diluted 1:75 with PBS and applied for 45 min. Control slides were incubated with nonimmune whole rabbit serum (Cooper Biochemical, Malvern, PA) diluted 1:1000 or, for the monoclonal antibody, with undiluted P$_3$ mouse myeloma cell line supernatant. The rabbit and mouse antibodies were visualized using an avidin-biotin peroxidase detection method (15) with kits purchased from Vector Laboratories (Burlingame, CA). Incubations were performed according to the manufacturer's directions. After several washes with tap water, the slides were counterstained with hematoxylin, dehydrated with ethanol, and rinsed in xylene, and coverslips were applied with Preservaslide (E. M. Science, Cherry Hill, NJ). Photomicrographs were taken with a Nikon Optiphot microscope.

Histological and immunohistochemical staining of cultured cells

After removing culture medium the coverslips were washed twice with PBS, fixed for 15 min with Bouin's solution, and washed twice with PBS at room temperature. The coverslips were kept immersed in PBS and stored at 4 C for up to 6 days before staining. Some coverslips were stained with hematoxylin, while other coverslips were processed as described above for immunohistochemical staining of tissue sections, excluding the treatment with xylene and replacing absolute methanol with PBS for the quenching step. After counterstaining with hematoxylin and dehydration with ethanol, the coverslips were carefully picked up with fine forceps, dipped in xylene until cleared, and mounted on glass slides with Preservaslide. The dilutions of primary antisera used in these studies are indicated in Table 3.

Time lapse cinematography

Percoll gradient-purified trophoblasts were diluted to a concentration of 8 × 10^5 cells/ml in DMEM-H-G containing 4 mM glutamine, 50 μg/ml gentamicin, and 20% (vol/vol) calf serum. Five milliliters of this suspension were placed into a Nunclon tissue culture flask with a surface area of 25 cm^2. The flask was placed on the stage of a Nikon Diaphot inverted phase microscope equipped with a Nikon incubator and temperature control unit, which was set at 37 C. The microscope's side port was fitted with a Bolex (Swiss Professional Movie Equipment Limited, New York, NY) H16M 16-mm movie camera controlled by a series 500 Cinephotomicrographic Apparatus (Sage Instruments, White Plains, NY). The light level was set with a Sage

FIG. 3. Transmission electron microscopy of Percoll gradient-purified cells. a) Two adherent immature cytotrophoblasts showing little rough endoplasmic reticulum, few lipid droplets and microvilli, and some glycogen (upper portion of righthand cell). Occasional coated pits were seen (arrows). b) A more mature cytotrophoblast exhibiting numerous microvilli, abundant smooth membrane vesicles, Golgi (g), many lipid droplets (L), branched segments of rough endoplasmic reticulum (R), and coated pits (arrow), a) ×10,140; b) ×10,800.
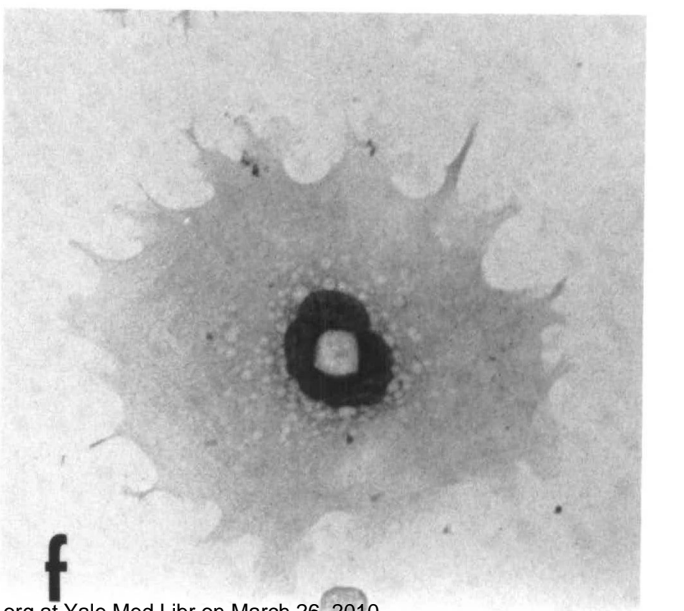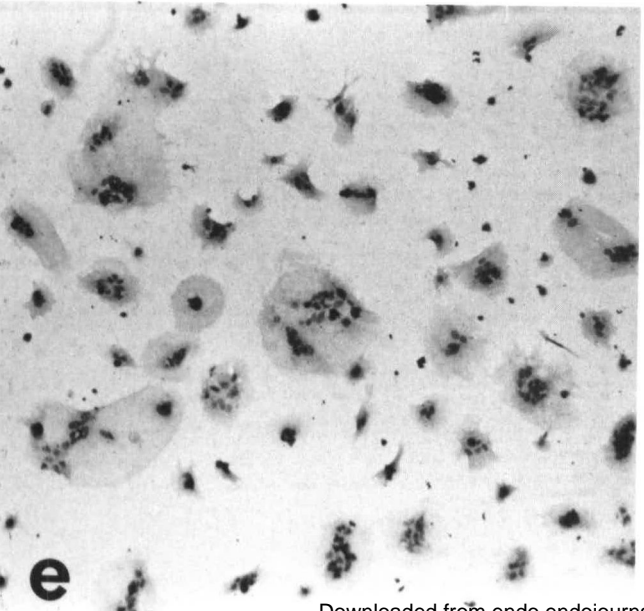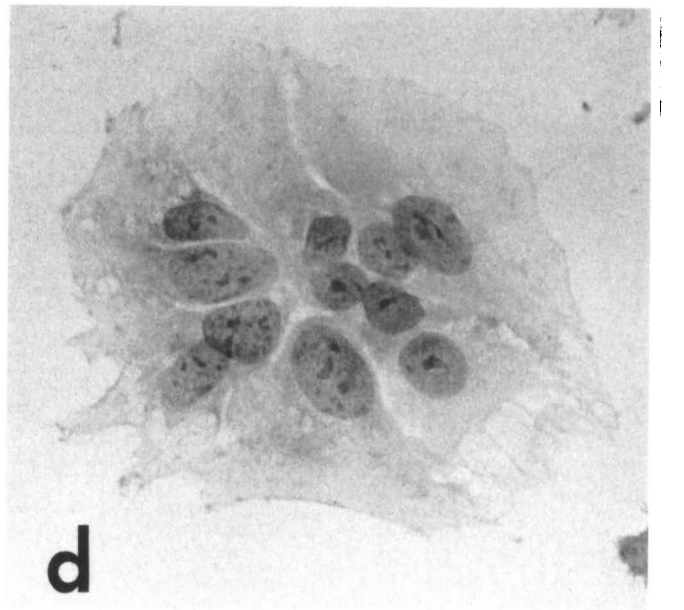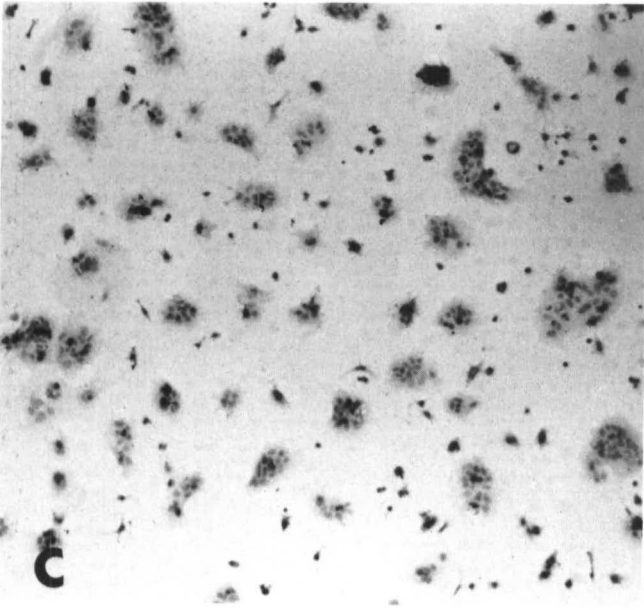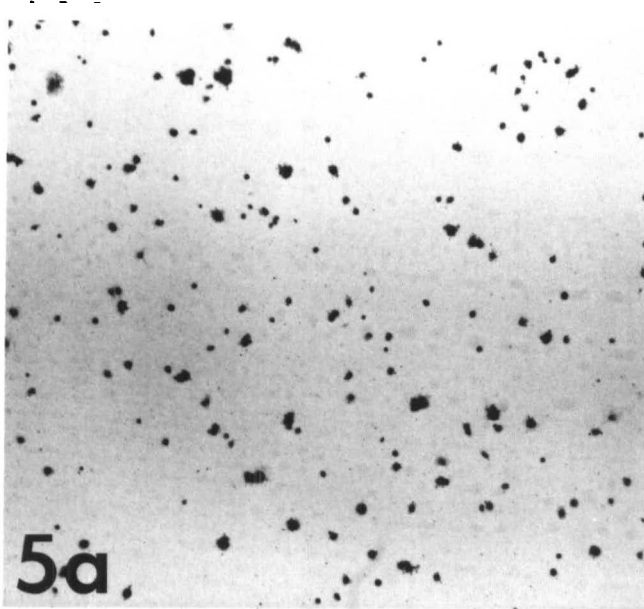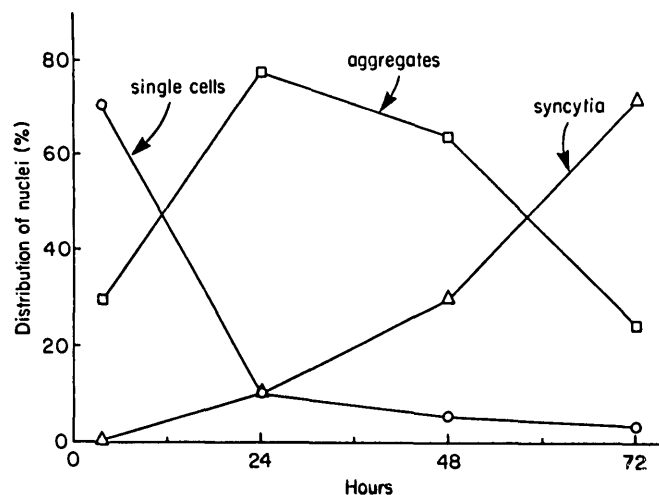
FIG. 6. Temporal changes in morphology of cultured trophoblasts. Cytotrophoblasts were prepared and cultured as described in Fig. 5. At the indicated time points, coverslips were fixed and stained. Beginning at a random point near the middle of each coverslip, 500 nuclei were counted in sequential high power fields and assessed as to whether they were in single isolated cells, single cells of an aggregate (2 or more cells attached to each other or single cells attached to presumed syncytia), or apparent syncytia.

Instruments Cinemicrography exposure meter. The cultures were photographed at a rate of four frames per min with a 0.5-sec exposure using Kodak Plus-X black and white reversal film (Eastman Kodak, Rochester, NY).

Microinjection of rhodamine-labeled α-actinin into cultured cells

Percoll gradient-purified trophoblasts were cultured for 5 days as described above. Coverslips were removed and transferred to 35-mm plastic petri dishes containing fresh medium. Presumed syncytial structures were microinjected with rhodamine-labeled α-actinin using a pressure microinjection system, as described by Sanger et al. (16). After recovery at 37 C in humidified 5% CO_2-95% air for 30 min, the coverslips were examined with a fluorescent microscope equipped with a SIT camera (Dage-MTI, Inc., Michigan City, IN), a highly sensitive video camera. Photographs were taken of the images produced on a high resolution video monitor.

Transmission electron microscopy

Percoll gradient-purified trophoblasts were resuspended into half-strength Karnovsky's fixative (17) at room temperature, pelleted at 1000 × g for 10 min and transferred to a 4 C refrigerator where they were kept overnight. The pellets were transferred to 0.1 M cacodylate buffer, pH 7.4, postfixed with

1% osmium-tetroxide for 1 h, dehydrated in graded ethanols and propylene oxide, and embedded in Epon. Silver sections were stained with uranyl acetate and lead citrate and examined at 50 kV with a Hitachi 600 electron microscope (Hitachi Corp., Tokyo, Japan).

DNA content analysis

The Percoll gradient-purified trophoblasts were fixed in 70% ethanol, stained with 4′,6-diamidino-2-phenylindole, and analyzed with an impulse cytophotometer (ICP-22A, Ortho Instruments, Westwood, MA), as described by Tsou et al. (18).

Other methods

Progesterone, estrogen, and hCG were quantitated by RIA. Progesterone was assayed as described by DeVilla et al. (19). Estrogens were quantitated with an antiserum that recognizes estradiol and estrone to the same extent (20). hCG was assayed using reagents purchased from Corning Medical (Medfield, MA). Protein contents of cells solubilized in 0.2% Triton X-100 in 1 N NaOH were quantitated by the method of Bradford (21) using BSA as a standard.

Results

Characterisics of the Percoll gradient-purified mononuclear cells

The goal of our initial studies was to isolate, from dispersed placental tissue, trophoblast cells which were free of contamination from formed blood elements, macrophages, and mesenchymal cells using Percoll gradients. Three distinct bands were visible in the Percoll gradients. Red blood cells were found at the base of the gradient, and pieces of villi, connective tissue, and small vessels were present at the top. In the middle of the gradient (density, 1.048–1.062 g/ml), we obtained a population of mononuclear cells (40–60 million/30 g starting placental tissue) which exhibited the structural characteristics as well as some biochemical features of trophoblasts. Under phase contrast optics, the cells collected from the middle band of the gradient appeared round, with the majority having a diameter of about 10 μm; occasional larger cells had a diameter of 20–30 μm. Trypan blue exclusion revealed that viability was greater than 90%.

Light microscopic examination of pellets of these Percoll gradient-purified mononuclear cells (Fig. 1) revealed a uniform population of cells, the vast majority of which

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FIG. 5. In vitro differentiation of cytotrophoblasts. Percoll gradient-purified cytotrophoblasts were cultured at a density of 8 × 10⁵ cells/ml fixed between 3.5 and 72 h after plating, and stained with hematoxylin, as described in Materials and Methods. a and b) 3.5 h, single cells and aggregates of two and three cells distributed evenly on the glass coverslip. The apparent intense staining of the cells is due to the fact that they are still spherical and have only begun to flatten out. c) 24 h, numerous aggregates interspersed among residual mononuclear cells. d) 24 h, representative aggregate composed of closely applied single cells. Note distinct spaces separating individual cells. e) 72 h, the multinuclear cells are now larger in size and appear syncytial. Fewer single cells are seen. f) 72 h, representative syncytial cell with several nuclei forming a central ring. Note the vacuolated cytoplasm surrounding the nuclei. a, c, and e) ×80; b) ×360; d and f) ×630.
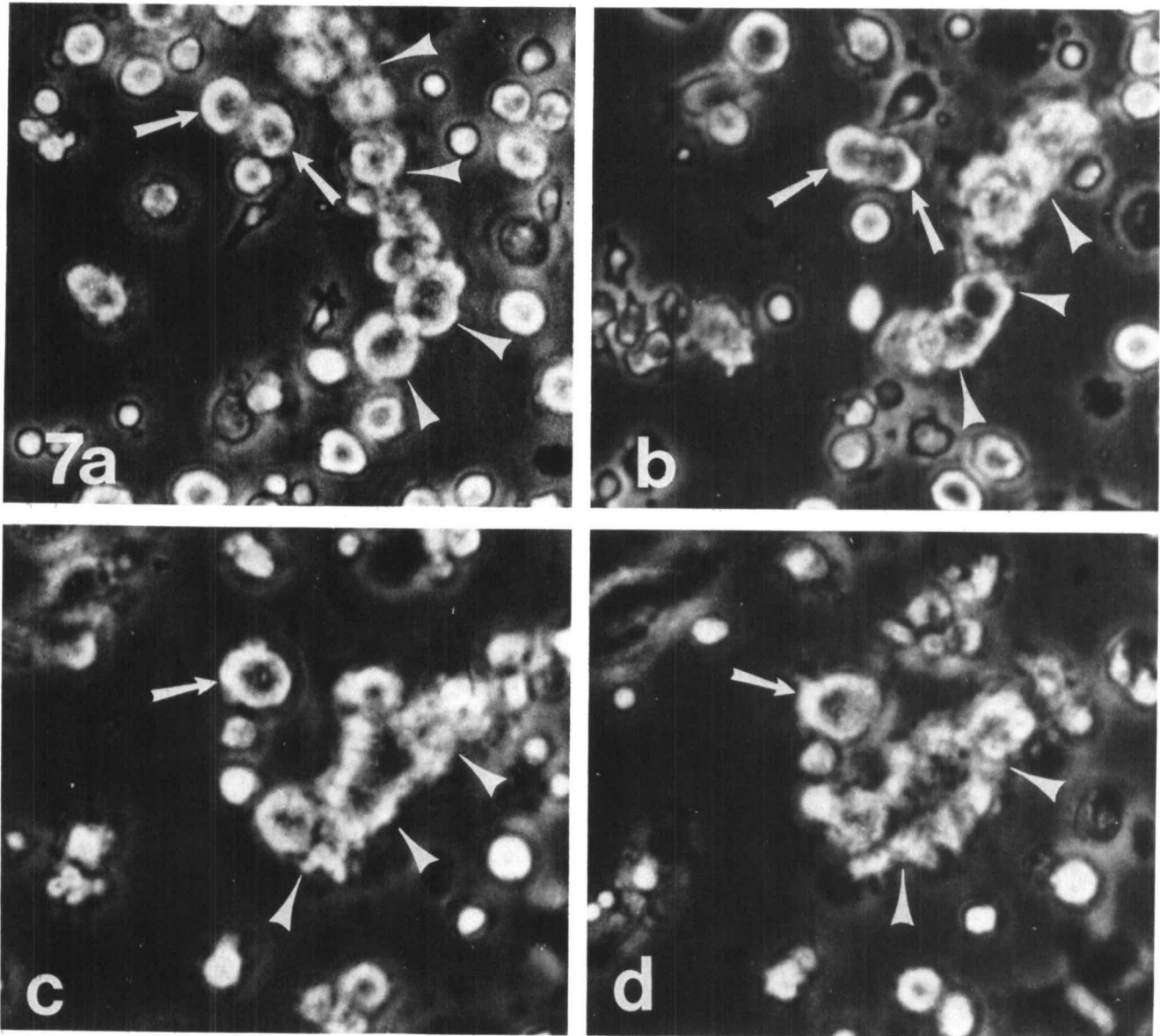
FIG. 7. Formation of aggregates and syncytia by cell fusion, as visualized by time lapse cinematography. Percoll gradient-purified cytotrophoblasts were diluted to a density of 8 × 10⁶ cells/ml, placed in a tissue culture flask, and photographed as described in *Materials and Methods*. Times are in hours:minutes after plating. a) 1:36, single cells are visible as a chain (*arrowheads*) and as a pair of cells (*arrows*). b) 3:24, the pair of cells now appears to have begun to fuse, while the chain of cells is beginning to aggregate (*arrowheads*). c) 5:36, the pair is now one cell (*arrow*), and the chain is becoming a single mass (*arrowheads*). d) 7:42, the recently fused pair (*arrow*) has migrated toward the mass of cells (*arrowheads*) and is beginning to fuse with it. Examination of this growing mass at the completion of the experiment (17:36) revealed a flattened structure with multiple central nuclei, consistent with a syncytia. Magnification, ×1400.

were mononuclear, with only occasional larger binucleated cells. Mitotic figures were noted with a frequency of 2 ± 1%.

DNA content analysis using 4′,6-diamidino-2-phenylindole-stained cells revealed the presence of an appreciable mitotic population. In one study, of 71,830 cells counted, 84.8% were in G_0 or G_1 (2N ploidy), 15.1% were in G_2 or mitosis, and 0.1% were in the S phase of the cell cycle. These data suggest that a significant fraction (15%) of the cells was actively dividing or ready to divide.

Previous studies have shown that hCG (22) and hPL

(23) are specifically localized to syncytial trophoblasts. In one previous report (24), SP_1 was claimed to be present in both cyto- and syncytiotrophoblasts. We examined the Percoll gradient-purified cells for markers of syncytial trophoblasts (hCG, hPL, and SP_1) as well as for other markers (cytokeratin, vimentin, and ACT) of nontrophoblast cells. Immunochemical studies performed on the starting placental tissue and the cells collected from the Percoll gradient are summarized in Table 1. An antiserum to hCG stained scattered syncytiotrophoblasts in the intact term placentae and occasional dispersed
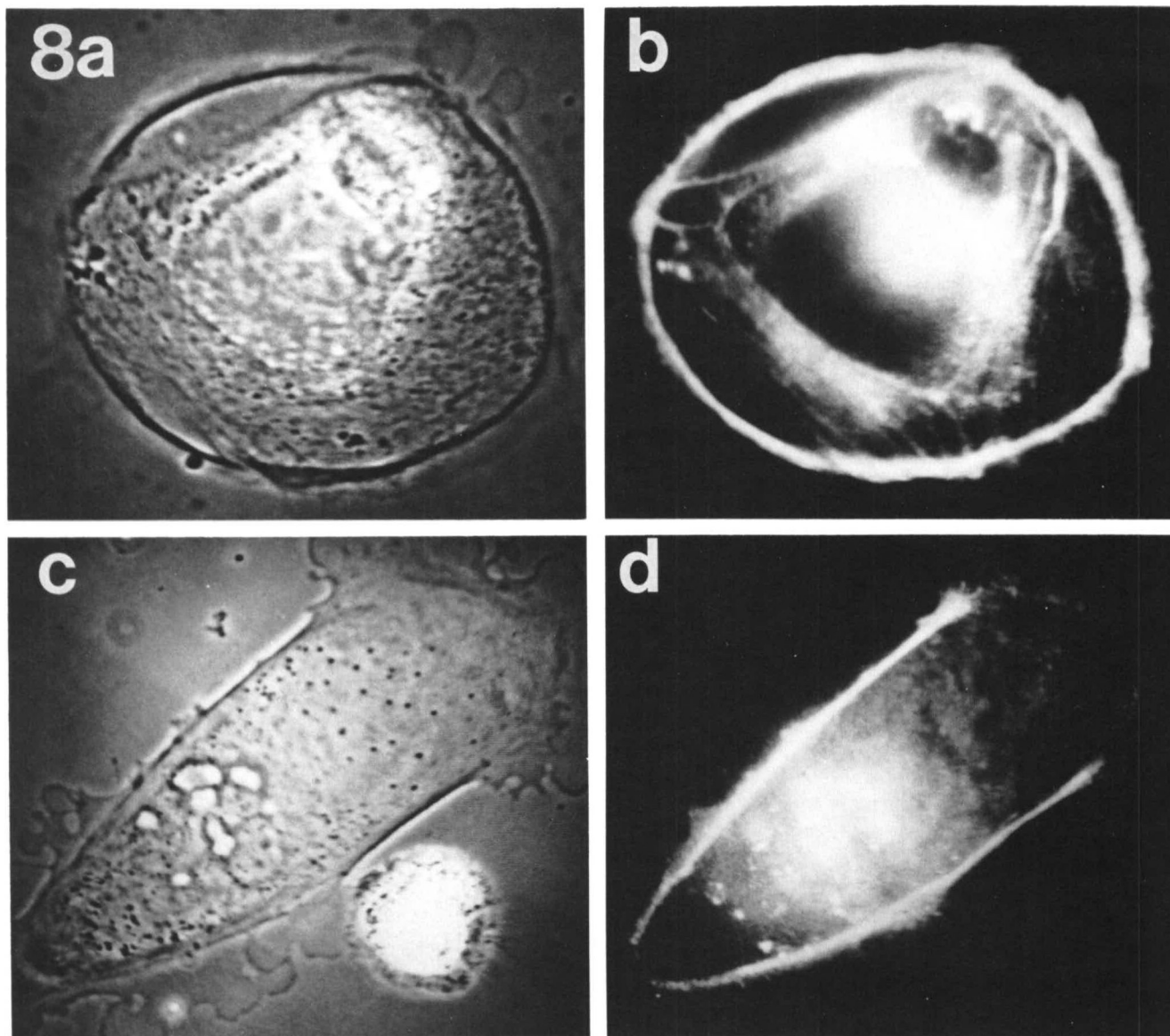
FIG. 8. Microinjection of rhodamine-labeled α-actinin into presumed syncytia. Percoll gradient-purified cytotrophoblasts were cultured on glass coverslips and microinjected with fluorescein-labeled proteins, as described in *Materials and Methods*. a) Phase contrast image of a syncytia. Nuclei are not well resolved, but are present in the central portion of the cell. b) Fluorescent image of the same cell shown in a. Fluorescein-labeled α-actinin has clearly diffused throughout the entire cytoplasm. Note the strong circumferential and perinuclear labeling as well as fine labeled filaments connecting perinuclear region and plasma membrane. c and d) Similar pair of micrographs of a second representative syncytium. a and b) ×1450; c and d) ×900.

cells purified on the gradient. At most, only 1–5% of the isolated cells displayed a positive reaction. An antiserum against hPL stained the syncytial trophoblasts of the starting term placentae intensely, but the cytotrophoblasts of the intact placenta and the isolated mononuclear cells did not react. Anti-SP$_1$ antisera also stained the syncytiotrophoblasts in the intact placentae, but not the placental cytotrophoblasts or isolated mononuclear cells. Thus, these three known markers for the syncytiotrophoblast, hCG, hPL and SP$_1$, did not stain the Percoll gradient-purified cells nor the cytotrophoblasts of intact placentae.

We discovered that a monoclonal antibody to lower mol wt cytokeratins, which identifies epithelial cells (25, 26), also stained the syncytial trophoblasts of the starting term placentae, but did not stain the Percoll gradient-purified cells. The monoclonal anticytokeratin did not stain the cytotrophoblasts in term placentae.

A polyclonal antibody was used to evaluate vimentin intermediate filament staining. Vimentin is one of a class of intermediate filament proteins known to be present in endothelial cells and fibroblasts (26). Antivimentin antibody strongly stained endothelial and mesenchymal cells of the chorionic villi of term placentae, but did not

TABLE 3. Distribution of immunocytochemical staining for trophoblast markers in cultured cytotrophoblasts

Antisera	Single cells			Aggregates			Syncytia		
	Negative	Positive	% Positive	Negative	Positive	% Positive	Negative	Positive	% Positive
NRS[a]									
3.5 h	323	0	0	177	0	0	0	0	0
24 h	57	0	0	373	0	0	46	0	0
48 h	37	0	0	219	0	0	244	0	0
72 h	16	0	0	90	0	0	394	0	0
hCG[b]									
3.5 h	383	4	1	106	2	2	5	0	0
24 h	46	7	13	337	59	15	34	17	33
48 h	19	17	47	86	302	78	30	46	61
72 h	14	7	33	19	81	81	202	177	47
hPL[c]									
3.5 h	352	3	8	145	0	0	0	0	0
24 h	47	1	2	376	8	2	45	23	34
48 h	7	0	0	336	18	5	134	5	4
72 h	15	3	17	92	30	25	329	33	9
SP$_1$[d]									
3.5 h	334	0	0	156	0	0	0	0	0
24 h	29	27	48	216	180	45	14	34	71
48 h	14	19	58	21	302	93	27	117	81
72 h	9	13	59	11	168	94	30	269	90

Percoll gradient-purified cytotrophoblasts were cultured on glass coverslips, fixed in Bouin's solution between 3.5 and 72 h after plating, and immunocytochemically stained to assess for cellular content of hCG, hPL, and SP$_1$, as described in *Materials and Methods*. Beginning at a random point near the center of each coverslip, 500 nuclei were counted in sequential high power fields, and each was assessed as to whether it was in a single isolated cell, single cell of an aggregate (2 or more cells attached to each other or single cells attached to presumed syncytia), or apparent syncytia. In addition, the cell was assessed as to immunoperoxidase positivity. Any discernible staining was considered positive. All nuclei in an aggregate were considered positive if any cell was positive. Likewise, all nuclei in a syncytia were considered positive if any part of the cytoplasm was positive.

[a] Nonimmune rabbit sera, diluted 1:1000.
[b] Diluted 1:800.
[c] Diluted 1:1000.
[d] Diluted 1:1000.

stain either the cyto- or syncytiotrophoblasts or the Percoll-purified cells (Fig. 2, a and b). This ruled out the possibility that our isolated cells were fibroblasts or endothelial cells.

To determine if the isolated mononuclear cells were macrophages (27), we performed immunochemical staining for ACT, a known marker for macrophages (28). Anti-ACT antibody stained only mesenchymal cells in the villi of the starting term placentae, but did not stain the isolated mononuclear cells (Fig. 2, c and d). Therefore, the isolated cells are not macrophages.

Transmission electon microscopy of freshly isolated Percoll gradient-purified cells revealed that cells of both sizes (10 and 20–30 μm) had similar fine structural appearances, typical of trophoblasts. However, the population consisted of cells in various stages of differentiation. Cells were seen which appeared immature (4) (Fig. 3a), containing little rough endoplasmic reticulum, few lipid droplets and microvilli, and some glycogen. Other cells (Fig. 3b) had numerous microvilli, many coated pits, abundant smooth membrane vesicles, well developed Golgi, many lipid droplets, branched segments of rough endoplasmic reticulum, and scattered multivesicular bod-

ies. These features are characteristic of mature cytotrophoblasts (4). Occasional cells were adherent and formed intermediate junctions, and rarely, cells were seen with internalized desmosomes. No cells exhibiting the structural features of fibroblasts or endothelial cells were identified. Cells with the structural features of macrophages were rarely seen.

To characterize the functional activities of the isolated mononuclear trophoblast cells, we studied their steroidogenic activity in short term incubations. Net synthesis of progesterone, defined as progesterone present in cells and media at the termination of the incubation minus the progesterone content of cells and media at zero time, averaged 1 ng/10^6 cells·4 h and progressed at a linear rate (Fig. 4). Progesterone synthesis was increased 2- to 8-fold by the inclusion of 20 μg/ml 25-hydroxycholesterol in the medium. This hydroxysterol is more soluble in aqueous medium than is cholesterol and readily enters cells; its metabolism by steroidogenic cells appears to be limited by the level of cytochrome P-450$_{scc}$ (29). Hence, it can be used to measure the maximum steroidogenic potential of cells. The fact that 25-hydroxycholesterol markedly increased progesterone synthesis in the cells

suggests that the cells may have been substrate limited when incubated in Minimum Essential Medium alone. In the presence of 20 μg/ml 25-hydroxycholesterol, progesterone synthesis was totally inhibited by the addition of 100 μM aminoglutethimide, an inhibitor of the cholesterol side-chain cleavage enzyme.

The isolated mononuclear trophoblast cells synthesized estrogens during short term incubation (Table 2). The formation of estrogens was increased greater than 20-fold by the addition of exogenous androstenedione (1 ng/ml). This is consistent with the known biosynthetic scheme for estrogens by the human placenta which holds that androgens, primarily formed by the fetal adrenal glands, are requisite substrates (30).

Differentiation of mononuclear trophoblasts into syncytial trophoblasts in culture

When the isolated mononuclear trophoblasts were placed into culture, there was a striking change in cellular morphology during the first 24 h (Fig. 5, a–d). After 3.5 h of incubation, mononuclear cells and aggregates of two or three cells were uniformly distributed on the glass coverslips (Fig. 5, a and b). After 24 h, numerous aggregates were identified interspersed among residual mononuclear cells (Fig. 5c). Most of these groups were composed of cells with clearly visible cell membranes separating the nuclei (Fig. 5d). Other multinucleated groups appeared quite different (Fig. 5f). These other multinucleated groups had centrally placed nuclei with no intervening membranes visible, and they appeared to be, therefore, syncytial groups. At 48 h, more aggregates and putative syncytia were noted, with fewer single cells. Cultures at 72–120 h showed more and larger syncytial groups (Fig. 5, e and f). In addition, after 72 h, the syncytia showed increasing amounts of vacuolated cytoplasm.

Quantitative counts of nuclei present in single cells, aggregates (two or more separate cells attached to each other or single cells attached to presumed syncytia), or apparent syncytia (Fig. 6) revealed a temporal change in cellular morphology consistent with a process of cell aggregation followed by syncytial formation. Single cells dominated at 3.5 h, but quickly dropped off at 24 h. Aggregates peaked at 24–48 h, while syncytia increased to a maximum of 72% at 72 h. In other, similar experiments, observations made 120 h after plating revealed that at least 90% of the nuclei were found in syncytia. Most aggregates contained 4–8 cells, and most syncytia contained an average of seven nuclei, but syncytia with as many as 30 nuclei were noted. There was no appreciable nuclear replication, as evidenced by the lack of mitotic figures. In over 60 coverslips examined from 4 separate experiments, only 1 mitotic figure was observed

of some 10,000 nuclei counted. Since mitotic events were exceedingly rare, and syncytia were observed as early as 24 h after plating, which is too soon for appreciable nuclear replication, our observations are consistent with the concept of syncytia formation through the process of fusion (2–5), rather than through endomitosis (6–8).

To verify that cell fusion occurred, we performed time lapse cinematography on cultures between 1 and 18 h after plating. These films clearly demonstrated single cells aggregating and fusing with each other and joining with larger groups to form larger aggregates and syncytia (Fig. 7). After aggregation, some cell masses were seen to round up and then flatten out again. These groups then appeared to have centrally placed nuclei, characteristic of syncytial groups.

Although these cellular groups appeared to be true syncytia under phase optics and after hematoxylin staining (Fig. 5f), we felt it was crucial to prove that these large cellular masses were truly syncytial and not simply closely adherent single cells. We chose to microinject the presumed syncytial structures with rhodamine-labeled α-actinin as a probe because it is large (mol wt, 190,000) and cannot be transported through cellular junctions. In addition, it binds to actin (16) and would be able to label the stress fibers of the putative syncytia and teach us something about their cytoplasmic organization.

Microinjection of fluorescently labeled α-actinin into presumed syncytial structures formed after 120 h in culture demonstrated that the labeled protein was capable of diffusing throughout the entire cytoplasm of the syncytial groups (Fig. 8). This experiment also revealed the cytoplasmic organization of the actin filaments in the syncytial groups. Actin appears to run circumferentially near the plasma membrane and is concentrated around the central nuclear mass.

Temporal pattern of the appearance of hCG, hPL, and SP$_1$ in cultured trophoblast cells

To assess whether the syncytiotrophoblasts formed *in vitro* were functional, we examined cultures between 3.5 and 72 h after plating for the presence of hCG, hPL, or SP$_1$ (Table 3 and Figure 9). At 3.5 h, the trophoblasts, which were still rounded and just beginning to adhere to the coverslips, demonstrated little cytoplasmic staining for hCG (1% of cells positive), hPL (0.8% positive), or SP$_1$ (0% positive). After 24 h, when cell aggregates dominated, increasing numbers of these aggregates contained positively staining cells, especially for hCG and SP$_1$. Of the three markers studied, SP$_1$ appeared to be expressed to the greatest extent in the time period examined. hPL, on the other hand, was seen in a smaller fraction of cell forms at all times examined. At 72 h, 94% of nuclei in aggregates and 90% in syncytia were associated with
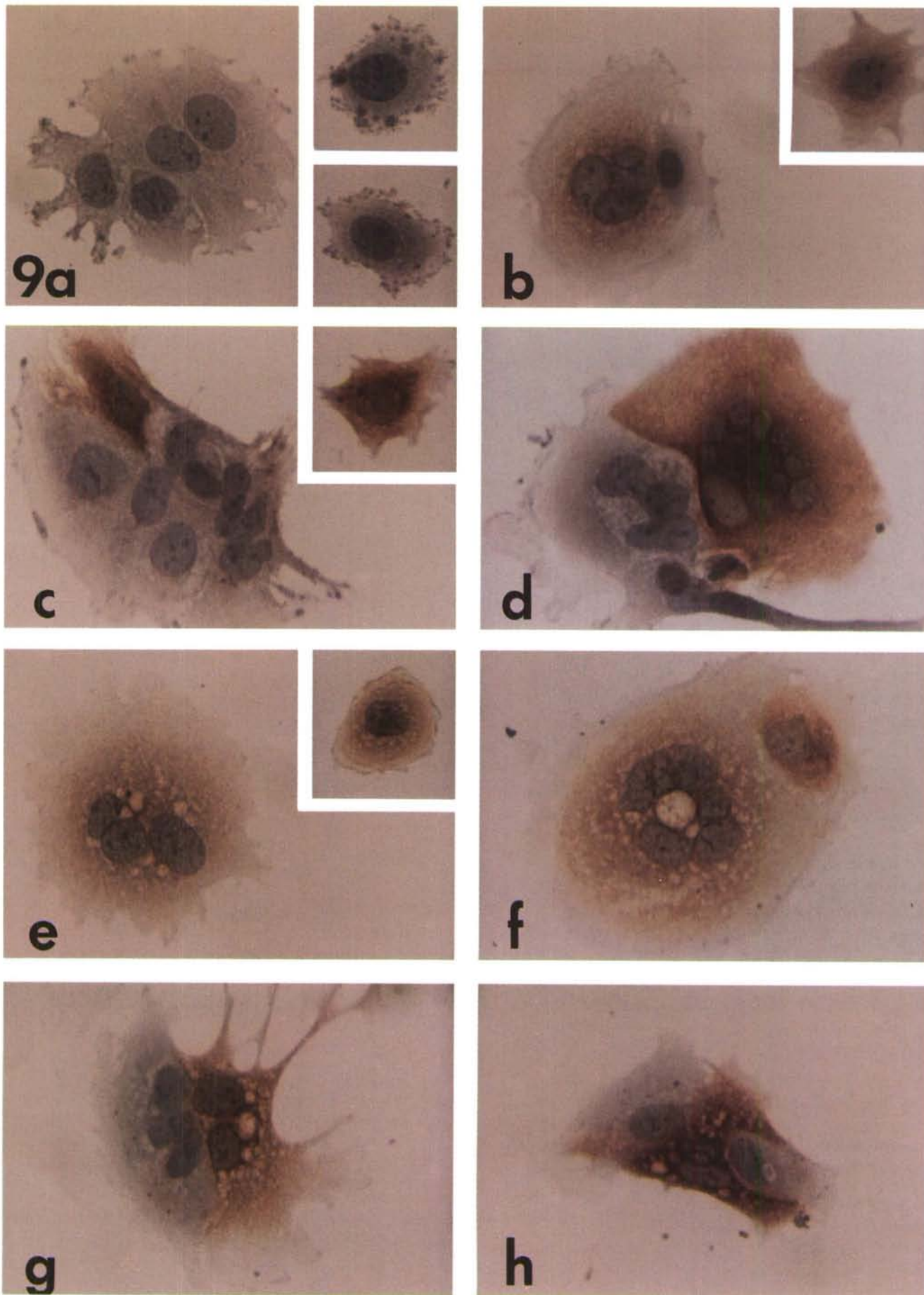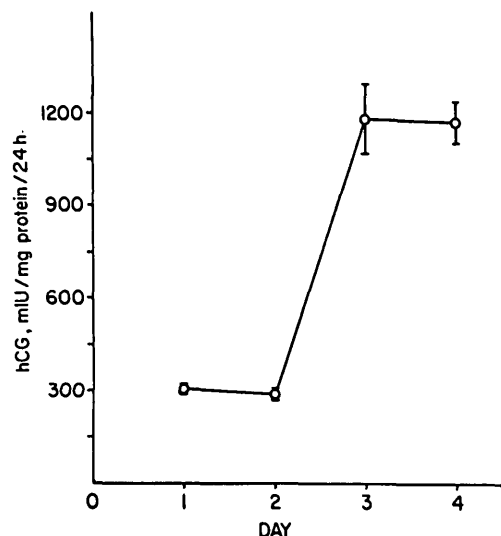
FIG. 10. hCG secretion by trophoblasts in culture. Percoll gradient-purified cytotrophoblasts were cultured as described in *Materials and Methods*. At the designated times, culture medium was removed and quick frozen, and assayed at a later time for hCG by RIA. After removal of medium on day 4, the cells were released by brief exposure to 0.2% trypsin in versene buffer, pelleted, treated with 0.1 N NaOH-0.2% Triton X-100, and assayed for protein. hCG values were normalized to day 4 protein content. At the time of plating, there was no detectable hCG in the culture medium. Values are the mean ± SE from five replicate cultures.

structures that stained positively with anti-SP$_1$ antibody. For hCG, the percentages were lower (81% and 47%, respectively). Finally, only 25% of the nuclei of aggregates and 9% of syncytia were associated with detectable hPL. Parallel measurements of hCG secreted into the media during this same time period revealed increased secretion of this hormone with time in culture (Fig. 10). The lag period in secreted hCG is consistent with the time course of appearance of hCG-positive cells stained with anti-hCG antibody (Table 3).

Representative immunoperoxidase positive cells are shown in Fig. 9. Single cells were seen that were either negative or positive for the markers studied (Fig. 9, b–e); cells within aggregates were also either positive or negative (Fig. 9c), and entire syncytia were positive or

negative (Fig. 9, b–h). In addition, mixed forms were observed which appeared to represent transition stages in the formation of syncytia (Fig. 9, b and h). In Fig. 9f, an hCG-positive syncytia is shown which appears to have had recently incorporated an hCG-positive single cell.

Discussion

A primary goal of this work has been to develop a method that could reproducibly generate a homogeneous population of cytotrophoblasts. We achieved this aim by adding a Percoll gradient centrifugation step to a well established method for enzymatic dispersion of human placenta (13). We have shown that the preparation of mononuclear cells isolated at a density range of 1.048–1.062 g/ml reveal structural features consistent with the range exhibited by cytotrophoblasts in term chorionic villi (4, 5). These cells are not fragments of syncytiotrophoblasts, since they do not stain for hCG, hPL, SP$_1$, or low mol wt keratins as do syncytial trophoblasts of term placentae. The cells do produce progesterone, increase progesterone synthesis up to 8-fold in the presence of the exogenous substrate 25-hydroxycholesterol, and produce estrogens when supplied with androstenedione as a precursor. Moreover, the cell preparations are not contaminated to any significant extent with endothelial cells, fibroblasts, or macrophages, as demonstrated by the lack of staining with antivimentin and ACT antibodies.

We have demonstrated that the majority of our Percoll-purified cytotrophoblasts consistently transform into aggregates, followed by syncytial formation within 24–48 h after plating. Previous studies using dispersed placental cells in monolayer culture have not noted this dramatic morphological change (10). Recently, Lobo *et al.* (31), also using the dispersion method of Hall *et al.* (13), added a filtration step through a 100-mesh screen sieve and exposure to ammonium chloride to lyse erythrocytes. They cultured their cells in medium 199 supplemented with 10% fetal bovine serum and found that with time, the percentage of multinucleated cells increased to

FIG. 9. Localization of hCG, hPL, and SP$_1$ in cultured trophoblasts. Percoll gradient-purified cytotrophoblasts were cultured, fixed between 3.5 and 48 h after plating, and immunocytochemically stained for cellular content of hCG, hPL, and SP$_1$, as described in *Materials and Methods*. All micrographs, ×750. a) Controls: cultured trophoblasts were stained with nonimmune rabbit sera at 3.5 and 24 h. *Upper inset* shows representative single cell at 3.5 h. *Left micrograph* shows a binucleated cell with two mononuclear cells adherent to its *lower lefthand border* at 24 h. *Lower inset* shows single cell at 24 h. b) hCG staining at 24 h. Trinucleated syncytium showing cytoplasmic hCG reactivity. Note syncytial cytoplasm partially surrounding a negative monnuclear cell. *Inset*, Representative mononuclear cell staining for hCG. c) hPL staining at 24 h. Multicellular aggregate containing one cell strongly staining with anti-hPL. *Inset*, Representative mononuclear cell containing hPL. d) hPL staining at 24 h. Multinucleated syncytium staining strongly with anti-hPL. Adjacent to this cell is a negative trinucleated syncytia. e) SP$_1$ staining at 24 h. Trinucleated syncytium showing cytoplasmic SP$_1$ reactivity. *Inset*, Representative mononuclear cell staining for SP$_1$. f) hCG staining at 48 h. Multinucleated syncytium exhibiting cytoplasmic hCG reactivity. Note the central clear vacuole, possibly representing lipid. Positively staining area in *upper righthand portion* of syncytial cytoplasm appears to be a recently incorporated positive single cell. Compare this to the partially incorporated cell in b. g) hPL staining at 48 h. Binucleated cell staining with anti-hPL adjacent to trinucleated negative cell. h) hPL staining at 48 h. Binucleated cell in center exhibits strong cytoplasmic hPL reactivity. Two negative mononuclear cells are seen adjacent to this cell. The mononuclear cell on the *right* appears to be in the process of incorporation into the syncytium.
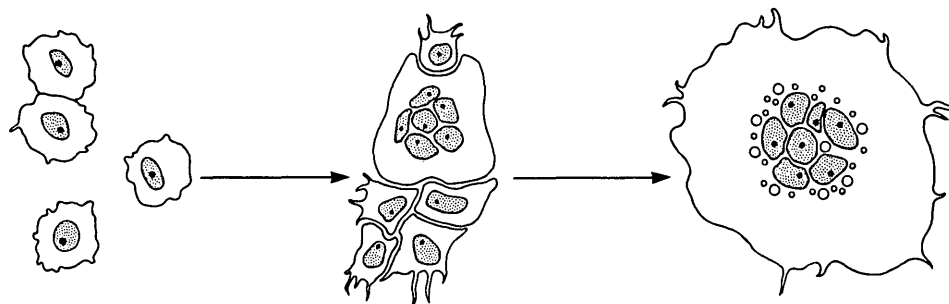
FIG. 11. Model of syncytiotrophoblast formation in culture. Initially, the isolated cytotrophoblasts are mononuclear single cells with some two and three cell aggregates. At 24 h, the dominant form is the multicellular aggregate. Although fewer in number, syncytia can also be seen at 24 h. After 24 h, increasing numbers of syncytia are seen until eventually they become the dominant form. It appears, therefore, that the cytotrophoblasts migrate toward each other to form aggregates and subsequently fuse with each other to form syncytia.

approximately 50% by 72 h in culture. The biochemical studies performed by Lobo et al. (31) are consistent with our observations, which reveal an increase in hCG secretion during the initial days of culture, but examination of their photomicrographs suggests that the multinucleated cells represent aggregates of single cells rather than true syncytia, since the nuclei appear to be evenly spaced from one another and not crowded into central portions of large cells (see Fig. 5, e and f for comparison). The mechanisms of syncytial trophoblast formation have been debated in the literature. Elegant work has been reported which strongly supports the notion that of the two types of trophoblasts, only the cytotrophoblasts are mitotically active, and they form the syncytium by fusion (32–34). Ultrastructural studies on intact placenta have shown syncytia with intracytoplasmic remnants of desmosomes and cell membranes, suggestive of a recent fusion event (2, 3). Although attractive, direct proof of the fusion hypothesis has been lacking. Alternatively, other workers have argued that endomitosis in the cytotrophoblast is the fundamental mechanism by which a syncytium is formed (8). On the basis of observations made under phase optics, Cotte et al. (6) and Stromberg et al. (7) suggested that syncytia form in cultured dispersed placental cells by endomitosis. Although the cytotrophoblasts we have isolated from the term placentae clearly have the potential to replicate DNA, as evidenced by the presence of mitotic figures in histological preparations and by DNA content analysis, evidence for significant nuclear replication in culture (mitotic figures) was not obtained. Moreover, from our time lapse cinemotography, it is evident that cytotrophoblasts migrate toward each other to form agregates, which eventually form syncytia through fusion. Our cinematographic observations suggest the model presented in Fig. 11. A previous report using time lapse cinematography of putative human trophoblasts in vitro (35) did not describe similar events. These investigators may have been unable to observe cell fusion, possibly because they employed a

heterogeneous population of cells, only some of which may have been cytotrophoblasts. Although we cannot completely dismiss the possibility that endomitosis contributes to syncytial formation, our findings clearly favor cell fusion as the primary mechanism.

To assess differentiation of trophoblasts in culture, we studied the acquisition of cytoplasmic staining for hCG, hPL, and SP_1. With increasing time in culture, more cells stained positively for hCG, hPL, and SP_1. Furthermore, our data reveal a differential and sequential acquisition of syncytiotrophoblast markers. Hoshina et al. (36), using in situ hybridization techniques to localize hCG and hPL mRNAs in choriocarcinomas, demonstrated that hCG mRNA was expressed in less differentiated cytotrophoblastic elements, while hPL mRNA was seen in more differentiated trophoblastic structures. These data, therefore, may explain the differential expression of SP_1, hCG, and hPL in our cultured trophoblasts. As the Percoll-purified cytotrophoblasts were cultured, they differentiated toward a syncytiotrophoblast phenotype and synthesized these products in a predetermined sequence: SP_1, hCG, followed by hPL. Our current study was not designed to detect cells that synthesized more than one product. Clearly, it would be of great interest to know whether these cultured trophoblasts can make more than one of these products at a time, or if they make them sequentially.

Careful examination of immunochemically stained cultures occasionally revealed single cells, isolated or part of aggregates, that were positive for hCG, hPL, or SP_1. These observations indicate that in this in vitro system, the syncytial state is not necessary for these trophoblast products to be expressed.

Our observations of the formation of syncytiotrophoblasts in culture parallel in many aspects the formation of myotubes from myoblasts (37). The myoblast system begins with mononuclear cells and ends with syncytial myotubes. Holtzer and colleagues (37), studying the transformation of myoblasts to myotubes, have suggested

that "Fusion does not trigger, it is but one of the consequences of the differentiation program of the myoblast." These workers have demonstrated that: only mononuclear myoblasts replicate; after a set number of divisions, mononuclear cells eventually become postmitotic and only then begin to make the unique mRNAs necessary for the synthesis of muscle-specific contractile proteins; and postmitotic mononuclear myoblasts, as part of their differentiation program, fuse to form the end-stage syncytial myotubes. We predict that the principles elucidated through the study of the myoblast system will also operate in the human trophoblast system because we have demonstrated that features of the trophoblast system parallel those of the myoblast system. These features include: a population of the mononuclear trophoblasts which are mitotically active, the mononuclear cells show a spectrum of structural features ranging from immature to mature trophoblasts, some mononuclear trophoblasts express markers of the differentiated syncytiotrophoblast (hCG, hPL, and SP_1) and syncytia form by fusion of mononuclear trophoblasts.

This trophoblast system should allow us to further explore the mechanisms that govern trophoblast differentiation, syncytial trophoblast formation, and endocrine activity.

Acknowledgments

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