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A Method for Obtaining Monolayer Cultures of Human Fetal Cells from Term Placentas.* (26689)

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The human placenta at term is considered by most authorities to be a senescent organ and histological preparations show extensive fibrinoid degeneration, calcification and infarction(1,2,3,4). In previous years investigators have reported failures in their attempts to grow cells from cultures of the villi of mature placentas(5,6). More recently Stewart, Sano and Montgomery(7), using explant cultures, obtained an outgrowth of fibroblasts from the villous portion of the mature placenta, but they were unable to grow the trophoblast or to detect in the growth medium any of the gonadotrophic hormone that is produced by the trophoblast *in vivo*. However, we found, while studying cultures of cells from placentas procured at time of therapeutic abortion, that it is also possible to obtain vigorous healthy cultures of a mixed cell type from the villi of mature placentas. In some cases chorionic gonadotrophin was demonstrated. Because of the shortage of human embryonic tissue for investigative work, the possibility that the villous portion of the mature placenta might provide an additional source of fetal cells was further explored. This paper describes a uniformly reliable technic for obtaining monolayer cultures of human fetal cells from the chorionic villi of mature placentas and describes the methods used to identify these cells as being of fetal origin.

Methods and materials. (A) *Tissue culture.* Mature placentas from 14 male and female pregnancies were collected aseptically at birth. Most of these were delivered vaginally. Individual cotyledons were identified on the maternal surface, excised and placed on a sterile towel with the fetal surface up. The membranes were removed exposing fetal tissue, and a layer containing chorionic villi

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5 to 8 mm thick was dissected from the central portion of each cotyledon. This material was pooled and an aggregate of approximately 25 g was washed 5 times in saline D2(8) to remove the blood and maternal elements. Random samples of these washed villi were taken for histological examination. The washed tissue was then transferred to a 250 ml Erlenmeyer flask with 45 ml of 0.03% trypsin (Nutritional Biochemical 1:300) in saline D2 adjusted to pH 7.0 and vigorously agitated at 37°C by means of a teflon-covered magnet over an insulated magnetic mixer. When the solution became turbid aliquots were taken for cell counts every 15 minutes until most of the cells were found to occur singly or in very small clumps. Length of trypsinization varied from 45-105 minutes. The suspension was then filtered into a beaker covered by a single layer of lint-free gauze. All of the tissue residue was recovered from the gauze and fixed for histological sections. The filtrate was centrifuged in a 50 ml round bottom tube at 800 rpm for 8 minutes and the supernate discarded. The spun cell sediment was suspended in 30 ml of saline D2 by gentle pipetting and a cell count made. The cell suspension was centrifuged again and resuspended in growth medium to a final concentration of 4.5×10^5 cells per ml. Growth medium consisted of 80% NCTC 109‡ and 20% fetal bovine serum§ with penicillin 100 units/ml and streptomycin 50 gamma/ml. Each 16×150 mm screw-capped roller tube containing a flying coverslip $6 \times 30 \times 0.2$ mm was inoculated with 2 ml of the cell suspension and incubated in a slanted stationary position at 37°C in a gassed incubator with 7% CO_2 in atmospheric air at 80% relative humidity. An average of 25-30 roller tube cultures was obtained with this technic. By removing larger amounts of tissue from the cotyledons, more cultures could be obtained. The medium was completely changed after 24 hours and then twice a week. After the first nutrient change, the medium removed was quantitatively assayed for chorionic gonadotrophin after the method of Delfs(9). The cultures, which were examined every other day, became confluent and ready for subculture in 10-14 days. (B) *Subculture method.* The growth medium was removed and the cells washed in saline D2 for 2-3 minutes. This was discarded. Each culture was washed again for 15-25 minutes at room temperature with 2 ml of saline D2 until the sheeted cells floated off the glass, at which time 2 drops of 0.25% trypsin in saline D2 were added to each roller tube. With gentle agitation a single cell suspension was obtained in 5 minutes. After a count was made of the pooled cell suspension, it was centrifuged. The spun cells were resuspended in growth medium to a final concentration of 4.5×10^5 cells/ml and roller tube subcultures made. (C) *Staining.* The samples of washed villi and the tissue residue after trypsinization were fixed in modified Davidson's solution(10) for 24 hours and then remained in 70% ethyl alcohol until paraffin sections were prepared and stained with hematoxylin and eosin. The flying coverslips were removed at random intervals during the first 10 days. Some were fixed in absolute methyl alcohol and stained with May-Grunwald and Giemsa to define cellular morphology. The remaining coverslips were stained to demonstrate sex chromatin. These were fixed in equal parts of 95% ethyl alcohol and ether, hydrolyzed in N HCl and stained in buffered thionin after the method described by Klinger(10). A quantitative analysis for sex chromatin was made of the primary cultures from each placenta by counting at least 200 interphase nuclei in each preparation. Overlapping nuclei or those showing degenerative changes were omitted. All other nuclei with a chromocenter were counted as sex chromatin positive. All the counts were made on primary cultures less than 2 weeks old because of reports that the number of chromatin positive nuclei decreases with subculturing(11). (D) *Controls.* Normal adult female buccal smears were used to insure the effectiveness of the thionin stain. The maternal placental surface of 2 male gestations, cultured in the manner described for the fetal surface, was used as a control to reflect the changes in the

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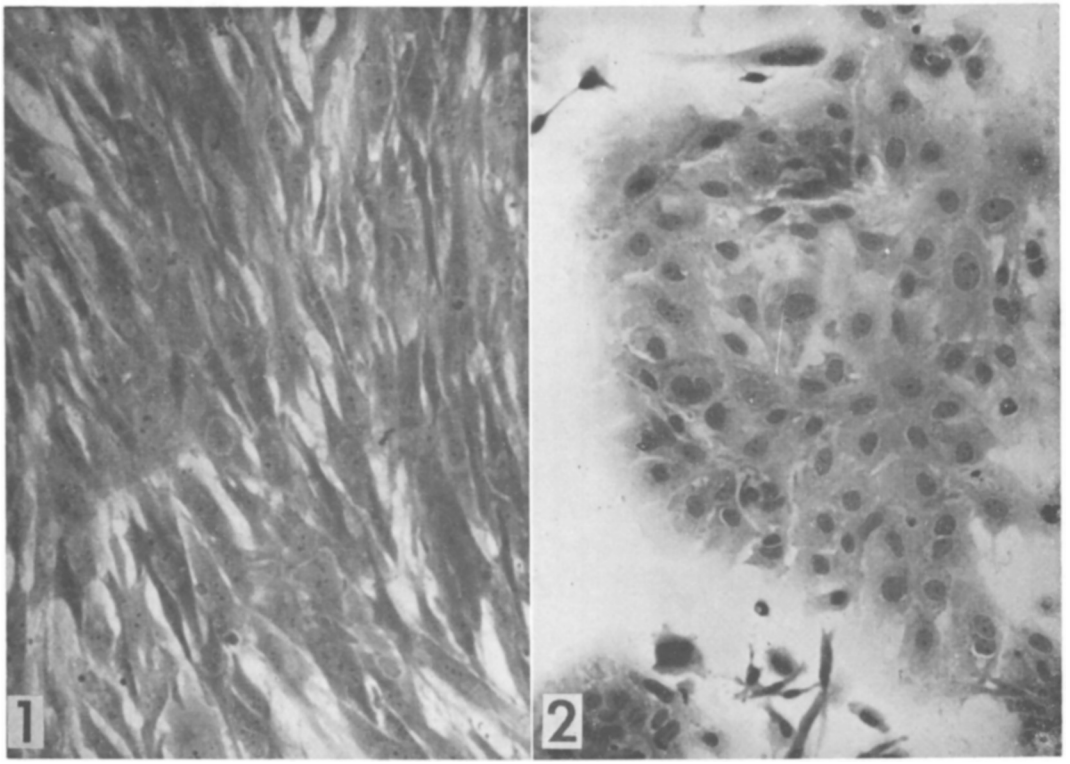

FIG. 1. A sheet of spindle cells. FIG. 2. A typical colony of epithelioid cells found in a monolayer culture of fetal placental cells from a mature placenta.

percentage of sex chromatin positive cells due to presence of decidual tissue.

Results. A total of 16 term placentas were processed for tissue culture. Fourteen were succesfully maintained in prolonged culture. Delay in obtaining one placenta after delivery and prolonged trypsinization of the other explained the 2 failures. Bacterial contamination was not a problem. The primary cultures of the fetal placental tissue showed a mixed cell population indistinguishable from the cell types previously described with cultures of therapeutic abortion material (12). These were (a) large and small spindle shaped cells (Fig. 1), (b) clusters of epithelioid cells which grew in a pavement like fashion (Fig. 2) and (c) occasional multinucleated giant cells. Following the first subculture, the spindle cells became the predominant cell and only a few colonies of epithelioid cells were seen. Chorionic gonodatropin hormone (HCG) was detected in the culture medium of 2 term placentas using the immature rat uterine weight method described by Delfs(9). Each ml of ether extracted medium (EEM) from one term placenta (TP I) after 3 days in culture contained 62.5 International Units (IU) of HCG. At 7 days none was found. The medium from TP III after 3 days in culture contained 277 IU of HCG per ml EEM and after 5 days in culture 700 IU of HCG per ml EEM. At 7 days and 10 days the assays were negative. These data suggest an *in vitro* production of HCG by the TP III cells, but this is not regarded as conclusive evidence. Careful examination of the histological sections of washed fetal placental tissue revealed no decidua. Multiple sections from the trypsinized fetal tissue of each placenta were also searched for decidua. Rarely was any found except for 3 specimens in which many single decidual cells and several small clumps of decidua were present. The decidual cells were clearly defined as large round intensely eosinophic cells with round nuclei containing a single prominent centrally located nucleolus. The percentage of nuclei

containing chromocenters was calculated for each placental culture. Less than 5% sex chromatin positive nuclei were found in all but 3 of the male fetal placental cell cultures. The counts of those 3 were 8%, 14% and 19% positive nuclei. The significance of this will be discussed. An average of 50% of the nuclei in the female fetal placental cell cultures contained sex chromatin. Examination of the histological sections of the washed and trypsinized tissue taken from the maternal surface of 2 male placentas revealed a somewhat greater number of decidual cells. An average of 35% of these cells in culture contained sex chromatin positive nuclei.

Discussion. The human placenta is largely composed of fetal tissue supported on a framework of maternal cells. The small amount of maternal tissue can be avoided by careful dissection. To confirm the fetal origin of the cell cultures obtained from the chorionic villi and to quantitate the number of maternal cells inadvertantly included. the sex chromatin pattern of the cell cultures was studied. Previous investigators have shown that the cellular elements of the fetal portion of the placenta have a sex chromatin pattern identical with the morphological sex of the fetus(13,14,15). Others report that the nuclei of the cells in the placental septum have a female chromatin pattern regardless of the sex of the fetus(16). The results of our studies agree with these reports. Fetal placental cell cultures from 11 male gestations contained less than 5% sex chromatin positive nuclei. This falls within the accepted percentage of male nuclei which contained chromocenters(17). Decidual cells were rare in the trypsinized material from which these cultures were obtained. The 3 fetal placental cell cultures which contained more than 5% sex chromatin positive nuclei were obtained from the same specimens in which greater numbers of decidual cells were found in the histological sections. Cultures of cells taken from the maternal surface of the placentas of male gestations also contained a greater number of nuclei with a female chromatin pattern. Histological sections of the trypsinized material showed a proportionate increase in decidua. It is clearly evident that with improved methods and materials, it is now possible to obtain human fetal cell cultures from the chorionic villi of mature placentas. The sex chromatin studies of the placental cell cultures from male gestations confirm the fetal origin of the cells. Placentas from both male and female gestations can be used to obtain these fetal cell cultures. Although sex chromatin studies are of no value in estimating the amount of decidua in placental cell cultures of female gestations, examination of histological sections of the trypsinized material will detect the inadvertant inclusion of maternal elements. These fetal cells provide investigators with an additional source of human embryonic tissues for experimental studies using tissue cultures.

Summary. Technics are presented for obtaining monolayer cultures of human fetal cells from the chorionic villi of mature placentas. Mixed cultures of epithelioid and spindle cells are obtained with these methods. The fetal origin of the cells in culture is confirmed with studies of the sex chromatin pattern of the nuclei and by examination of histological sections of the trypsinized tissue for the presence of decidua. Detection of chorionic gonadotrophin in the medium of 2 cultures of cells from mature placentas is reported.

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Dissociation of Uridine Diphosphate Glucose-Glycogen Transglucosylase from Phosphorylase Activity in Individual Muscle Fibers. (26690)

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The present concept of a cyclic glycogen metabolism in muscle(1) assumes the existence of different enzymatic pathways for glycogen synthesis and breakdown. The catabolic enzyme is represented by phosphorylase(2), and polysaccharide synthesis from glucose-1-phosphate is considered to depend on the uridine diphosphate glucose (UDPG)-linked pathway with the UDPG-glycogen transglucosylase reaction(3) as the final step.

Histochemical studies on the distribution pattern of these enzymes in resting skeletal muscle of the rat revealed a reciprocal relationship between phosphorylase and UDPG-glycogen transglucosylase activity in individual fibers. A different spatial arrangement of the two activities would be inconsistent with their operating as parts of a single enzymatic cycle.

Methods. Muscle tissue, excised from rats bled under ether anesthesia, was quenched in liquid oxygen. From tissue blocks oriented in a transverse or longitudinal plane fresh frozen sections were cut at 8 μ on a cold microtome (cryostat) at $-20°$ and mounted on coverslips. The activity of phosphorylase, branching enzyme (amylo-1, 4 $\rightarrow$ 1, 6-transglucosidase) and UDPG-glycogen synthesizing enzyme was demonstrated according to Takeuchi(4,5,6). The reaction for the last of these was carried out in the presence of .0005*M* glucose-6-phosphate. The glycogen content of untreated cryostat sections was assessed by the periodic acid-Schiff procedure after post-fixing the sections in Gendre's fluid. The methods employed for demonstration of various oxidative enzymes have been listed previously(7).

Results. A striking difference in enzymatic activity was noted in longitudinal and cross sections of muscles containing fibers of varying size. Both phosphorylase and branching enzyme were strongly active in fibers of large diameter(7). Conversely, high activity of UDGP-glycogen-transglucosylase was obtained in fibers belonging to the small ("red") type (Fig. 1, 2). Fibers of intermediate size showed varying degrees of activity with either type of reaction. Strong UDGP glycogen-transglucosylase activity was accompanied by high activity of oxidative enzymes, especially succinate and lactate dehydrogenases. In striated muscle of uniform fiber type (for instance, tongue, *M. levator ani*) little or no variation in enzyme activities was observed.

In contrast to findings in pigeon breast muscle(8) resting rat muscle showed intrinsic, diastase-labile, glycogen to be concentrated in the narrow fibers. In the same fiber type, polysaccharide formation was dependent mainly on the UDGP-transferase reaction.

The phosphorylase reaction was inhibited by substituting adenosine-5′-triphosphate