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Cytological Virological and Chromosomal Studies of Cell Strains From Aborted Human Fetuses.* (31037)

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Spontaneous abortion, usually without obvious cause, is a frequent occurrence in human pregnancies. To test the hypothesis that viral infections may play a part in the development of spontaneous abortion, a technique was sought to obtain dividing cells from human embryos that might be carrying latent viruses. We used a method developed by Jensen *et al,* for studying mouse tissues, in which cells could be obtained readily from organ explants. In the course of this work we collected cytological and chromosomal data on human fibroblast cell strains.

Materials and methods. Collection and preparation of specimens. Embryos were obtained from 2 sources: (A) surgical abortions performed in Scandinavia for social and psychiatric reasons, and (B) spontaneous abortions that occurred at the Philadelphia General Hospital and the Hospital of the University of Pennsylvania. The surgically removed embryos were placed in antibiotics containing Hanks' solution and shipped to us by air at a temperature of approximately 0°C. The spontaneous abortions were refrigerated in plastic bags without solution or antibiotics until collected, usually within 12 hours. Only those embryos which were expected to have viable tissues were studied. Aside from the decomposed external appearance, one of the best indicators of the embryo's condition appeared to be the physical aspect of the liver. All assays performed on embryos with friable and discolored livers were discarded, because the cells failed to grow.

Organ culture technique. The organ culture technique described by Jensen *et al*(1) was used: a grid of stainless steel mesh‖ was enclosed in a small Petri dish containing 10 ml of double strength Eagle's Basal Medium in isotonic Earle's solution with 10% calf serum; a small disc of open mesh paper (tea bag paper)** was moistened in the medium and applied to the top of the grid. Fragments of organs were cut into pieces about one cubic mm with a surgical blade and placed directly on the tea bag paper without being washed. Two explants were placed on top of each paper; the volume of the individual explants did not exceed 2 cu mm. The

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cultures were incubated at 37°C in a CO_2 incubator and the medium changed once a week. Cells migrated from the cut surfaces of the explant and dropped to the bottom of the Petri dish, where they multiplied to form colonies and, in some cases, confluent cultures.

Establishment of cell strains. If the colonies became confluent and covered the entire surface of the Petri dish, a cell strain was established by trypsinizing the cells and subcultivating them, either in Petri dishes or in milk dilution bottles at a 1:2 split ratio.

During the first trypsinization, the grid was removed and placed in a second Petri dish and new colonies proliferated. After establishment of the cell strain, the technique used was the same as that of Hayflick(2) for cultivation of human diploid cell strains, whereby cultures were passaged approximately once a week with 2-fold subdivision.

Cytologic studies. Cytologic studies were conducted either on the colonies of cells that developed on the surface of the Petri dish or on established cell strains. Preparations stained with May-Grünwald-Giemsa were obtained by placing coverslips on the floor of the Petri dish under the grid bearing the organ culture or under passaged cells.

Chromosomal technique. This technique was derived from Lejeune(3). All the chromosomal studies were done on coverslips placed on the floor of the Petri dish under the grid or in Leighton tubes or on Petri dishes inoculated with resuspended cells after the cell strain had been established.

The cells were pretreated with colchicine by adding one drop of a stock solution of "Colcemide" (Ciba) containing 25 μg/ml to 5 ml of supernatant medium with a syringe and 24-gauge needle. The culture was incubated at 37°C for 3½ hours. The coverslip was transferred, face up, to a Petri dish that contained a hypotonic solution and was kept at 37°C for 35 minutes.

The hypotonic solution was a mixture of one part calf serum, 10 parts distilled water, and sufficient hyaluronidase ("Widase," Wyeth) to give 2.5 USP units per ml of the mixture.

The concentration of serum in the hypotonic solution varied, depending on the density of the cells on the coverslip. When the density of the cells was high, the concentration of serum was lowered.

After hypotonic treatment, the coverslips were removed and put into a new Petri dish with the fixative and left for 45 minutes at room temperature.

The fixative consisted of 3 parts chloroform, one part acetic acid, and 6 parts absolute ethyl alcohol. The coverslips were then air dried and placed in a 1 N HCl solution at 60°C for 7 minutes so that the cytoplasm could be hydrolyzed. The coverslips were washed thoroughly in buffered water and stained with Giemsa solution diluted 1 to 10.

Results. These studies were performed from March to July, 1962, when 36 embryos were used, and again from November, 1963 to May, 1964, with 40 embryos.

Growth of cells from organ cultures. Each Petri dish was examined at least once a week with an inverted microscope. The time interval between the start of the organ culture and the formation of the first colonies of a few cells growing on the bottom of the Petri dish differed greatly from one embryo to another. In some cases, the colonies started at the end of the first week, while in other cases, they started only after 3 to 4 weeks of incubation. Most colonies grew well and after 3 weeks measured several millimeters in diameter.

The criterion of success of a culture was whether or not cell colonies developed after one month on the bottom of the Petri dish that contained the tissue-bearing grid. Cell growth from at least one tissue failed to occur only in 14 out of 76 aborted embryos: 8 from Scandinavia, 4 from PGH and 2 from HUP.

In the first series, the last 7 out of 26 embryos received from Scandinavia did not give viable cultures. The non-viability of cultures was probably due to the high external temperature during their shipment in July. Of the 12 received from HUP, 2 were lost by contamination and 2 failed to grow. Successful cultures, however, were obtained from 25 embryos (6 from the HUP and 19 from Finland).

Of the 40 aborted fetuses studied between

TABLE I. Cell Growth Under Organ Cultures.

Organ	Embryos studied from Nov. '63 to May '64 — No. of embryos studied	Confluent cultures	Cell colonies	No growth	Successful*	Embryos studied Mar. to July '62 Successful	Total
Pituitary	21	15	4	2	19/21	25/27	44/48
Lung	30	27		3	27/30	12/15	39/45
Skin	30	25		4	26/30	8/8	34/38
Kidney	14	10	4		14/14	6/7	20/22
Spleen	12	3	8	1	11/12	4/7	15/19
Thymus	15	11	2	2	13/15	2/2	15/17
Heart	8		1	7	1/8	1/5	2/13
Intestine	8	1	4	3	5/8	0/4	5/12
Liver	7		6	1	6/7	0/4	6/11
Thyroid	5	5			5/5	2/3	7/8
Salivary glands						5/5	5/5
Adrenals						2/5	2/5
Pharyngeal mucosa	2	2			2/2		2/2
Whole embryo	1	1				1/1	2/2
Cornea						1/1	1/1
Meningea						1/1	1/1
Tongue						1/1	1/1

* Denominator: No. of embryo studied; numerator: No. of cultures with successful growth.

November, 1963, and May, 1964, 13 were sent from Scandinavia, 20 came from PGH, and 7 from HUP. Successful cultures were obtained from 12, 16 and 7 embryos, respectively. Table I presents the results of organ cultures initiated with tissues from 60 embryos (31 from Scandinavia, 16 from PGH, and 13 from HUP). At least one organ culture from this group was successful.

There is a distinction between confluent culture and cell colonies: in the former case, the cultures came to confluence and could then be used to establish a cell strain, while in the latter case, only discrete colonies formed.

From these results it appears that, with the exception of heart organ cultures, most preparations resulted in cell growth on the glass. It was usually possible to obtain confluent cultures from such tissues as skin, lung, pituitary, kidney, thymus, thyroid, and pharyngeal mucosa.

The extremely low proportion of bacterial and fungal contaminations (2 of 76) in these organ cultures was noteworthy.

Establishment of cell strains. Table II summarizes the results of attempts to establish cell strains from the confluent cultures developed under the grids. While cell strains were easily established from skin, lung, pharyngeal mucosa and pituitary, it was difficult to establish strains from intestine, thymus and thyroid.

All the cell strains were composed of fibroblast-like cells. With skin, lung and pharyngeal mucosa organ cultures, the cells under the grid were already predominantly fibroblastic; in the case of other organ cultures such as pituitary, thymus and thyroid the cultures at first appeared to be epithelial, but after the first trypsinizations became fibroblastic.

All of the cell strains had the previously described characteristics(2) for human diploid cell strains.

Virological studies. Two types of speci-

TABLE II. Establishment of Cell Strains.

Organ	No. of embryos studied	Culture sucessful for: More than 4 splits 1:2	Fewer than 4 splits 1:2	Culture unsuccessful at 1st split
Skin	16	15	1	
Lung	12	10	2	
Kidney	5		4	1
Pituitary	5	3	1	1
Pharyngeal mucosa	4	4		
Intestine	4	1		3
Liver	3			3
Thymus	3	1	2	
Thyroid	3	1	2	
Whole embryo	1	1		

mens were tested in an attempt to isolate viruses from embryos. The test systems used in both types were primary vervet monkey kidney, primary human amnion, and human diploid cells (the WI-38 lung strain) (2).

In an attempt to detect latent viruses, the first type of specimen used was obtained from the cell cultures that became cell strains. No cytopathic effects were seen in any of the cells continuously cultured for periods ranging from one to six months. Tissue culture fluids obtained from cell strains cultured for 2 to 4 weeks were inoculated undiluted onto monolayers of the 3 tissue culture test systems. The test systems were maintained under Eagle's medium and 2% calf serum for 3 weeks before being discarded.

Suspension of cells derived from organ cultures were inoculated onto green monkey kidney cell monolayers, a technique described by Gerber and Kirschstein (4) for the transfer of cell-associated virus. All of these inoculations were negative.

The second type of specimen was the supernatant fluids from cultures which failed to grow. One might consider that the failure to establish a cell strain was due to a cytopathic effect. Tissue culture fluids were harvested over several weeks from organ cultures prepared from 5 embryos which yielded no cell growth from any culture. Inoculation of these fluids onto the test systems showed no evidence of cytopathogenicity.

When explants from a particular embryo gave both successful and unsuccessful cultures, the tissue culture fluids from unsuccessful explants were also tested for the presence of virus. Once more all attempts were negative. It is important to note that failure to grow cells from explants occurred in the same proportion in embryos from surgical abortions as in embryos obtained from spontaneous abortions.

We prepared organ cultures from 3 tonsils to test the sensitivity of the organ culture techniques for isolation of latent viruses when no cells grew from the explant. No cells grew in any of these cultures on the bottom of the Petri dish; however, in one case, 2 weeks after the beginning of the cultures, an adenovirus was recovered by passage on a sensitive cell system of the medium harvested.

FIG. 1. Multinucleated giant cells seen in explant culture from a spontaneous abortion.

Cytological studies. Multinucleated cells were observed in many of the organ cultures, including explants from spontaneous and surgical abortions. Typical giant cells are illustrated in Fig. 1. Pituitary explants, in particular, gave rise to multinucleated cells, but when the cells were seen, their presence was noted in other cultures from the same embryo. Several days after the beginning of the culture, numerous giant-like cells containing 3 to 20 nuclei appeared. They were usually observed for the first time about the 12th day, but occasionally appeared before the seventh or as late as the 25th day of culture. The formation of multinucleated cells did not, in the majority of cases, prevent the eventual outgrowth of fibroblasts and development of a diploid strain. In pituitary cultures, the following sequence of events was observed: small colonies of epithelial-like cells appeared below the fragments, and later degenerated, giving way to a population of fibroblasts. As mentioned above, the fluids harvested from these cultures were tested on different cell systems with negative results. Some of the supernatant fluids were also inoculated into animals—such as baby mice by intraperitoneal and intracerebral routes, and baby hamsters by subcutaneous and intraperitoneal routes—without the isolation of a transmissible agent.

Chromosomal studies. Chromosomal study of cell cultures from 18 embryos of 2 to 4 months gestation was undertaken. Of these 18 embryos, all of which were obtained during the second time period of this work, 12

were male and 6 female. Four out of the 18 were surgically aborted, and the rest were obtained from spontaneous abortions.

All of the cell strains were diploid with a normal karyotype of 46 chromosomes. In 2 cases, both spontaneous abortions, chromosomal breaks were observed. In a male embryo, 24 of 79 metaphases analyzed (30%), had true breaks or gaps of one chromatid or of the two chromatids. The distribution of these breaks was of a random type. In a female embryo, which was one of twins, breaks were observed in 11 of 49 metaphases or 22%. The other twin, a male, had a normal karyotype. In 5 cells these breaks were on chromosome 3, at the same region in one or both chromatids, while in 3 other cells a constriction was observed at the same region.

In the remainder of cell strains, the percentage of gaps was below 10%.

Discussion. In this study it has been demonstrated that it is possible to derive cell strains from organ explants of human tissues, using the simple method described by Jensen *et al.* This method could be useful when dealing with small amounts of tissue such as fetal organs. The strains derived seem to be similar in behavior to the human diploid fibroblast cell strains obtained from minced tissues by Hayflick and Moorhead.

It seems important to have techniques that permit the establishment of cell strains from different organs. Recent studies have shown that human diploid cell strains vary in their sensitivity to viruses. For example, we have shown(5) that the effects of rubella virus infection are related to the organ from which these cell strains were initiated. Recently Behbehani *et al*(6) found that cell strains derived from human atheromatous lesions seem to be particularly susceptible to rhinoviruses.

The failure to isolate viruses from the spontaneously aborted fetuses must of course be qualified by the fact that only cytopathogenic agents would have been detected. However, insofar as the results are negative, some support should be given to the view that human diploid cell strains are normally free of extraneous viruses, and they are, therefore, advantageous for the fabrication of vaccines and for studies on chronic viral infection in human cells.

The negative results do not entirely exclude the possibility that viral infection plays a role in spontaneous abortion because the abortion might be due to a secondary effect of viral infection of the mother that has occurred without passage of the virus to the embryo itself.

No abnormality of the karyotype was observed among the 18 embryos studied. The only aberration found was due to breakages in 30% and 22% of cells of two of them. These results were in accordance with the results of Makino *et al*(7), who found only 2 aberrations out of 135 embryos obtained from therapeutic abortions: one aberration was D Trisomic, and in the other, the cells were found to contain a high incidence of chromosome breakage.

Chromosomal aberrations were found in spontaneous abortions by Carr(8), Clendenin (9), Szulmann(10), Hall(11) and Thiede (12), but in each case, where chromosomal abnormalities were described, the specimen was pathologic and consisted of a degenerating embryo or of an empty sac without a trace of fetal tissue—the so-called blighted ovum. Moreover, these pathologic specimens led to abortion which occurred early in pregnancy, or before the third month. In our study, most of the specimens were obtained from abortions that occurred in the third month or later, and which produced normally developed embryos.

Summary. An organ culture technique was used to investigate the possibility that latent viruses are present in spontaneously aborted human fetuses. All attempts to isolate virus from 74 human embryos were negative. In the course of these studies, numerous cell strains were derived from human tissue, and cytological features of these cells are described. Multinucleated giant cells were frequently found, but chromosomal aberration in this material was infrequent.

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Activation of Factors XII (Hageman) and XI (PTA) by Skin Contact.* (31038)

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Blood coagulation can be initiated *in vitro* by contact with a foreign surface such as glass which activates Factors XII (Hageman) and XI (PTA)(1). Most known activating surfaces do not occur in the body and it is unknown whether similar reactions initiate *in vivo* coagulation. Recently stearic acid(2-6), uric acid(7) collagen and elastin(8) which are found *in vivo* have been shown to activate the Hageman and PTA factors. Evidence is presented below that blood contact with unbroken human skin results in accelerated clotting due to activation of the Hageman and PTA factors.

Materials and methods. Platelet-poor plasma was prepared without contact with glass or similar surfaces as previously described(6). Plasma deficient in Factors VIII, IX, XI or XII was obtained from patients with congenital deficiency of these factors. Celite exhausted plasma deficient only in Factors XII and XI was prepared by treating normal plasma with 20 mg celite per ml as previously described(6). Cephalin prepared as previously described(9) was used in a 1/100 dilution.

Coagulation was carried out in 10 × 75 mm glass tubes coated with siliclad (Clay-Adams). 0.1 volumes of plasma and cephalin were added to a silicone treated tube. The tube was inverted over an area of skin which had been carefully cleaned with ether, alcohol and then distilled water and the plasma-cephalin mixture was incubated in contact with the cutaneous surface for a variable time period. The tube was turned upright, 0.1 ml 0.025 M $CaCl_2$ was added and the tube re-inverted over the same cutaneous site so that the clotting mixture was again in contact with the skin surface. The time required to form a solid clot was measured from the time calcium was added. In the control experiments exactly the same procedure was carried out except that parafilm (Marathon, Wisconsin) was interposed between the clotting mixture and the skin surface during both the incubation and clotting periods. Each clotting time was recorded as the average of those obtained in 3 tubes.

Results. Incubation of normal plasma in contact with a cutaneous surface resulted in progressive shortening of the clotting time (Fig. 1). Most of the acceleration of clotting occurred during the first minute of incubation and after 5 minutes incubation an almost maximal effect was noted. Skin surfaces in various sites exerted different degrees of clot promoting activity—the palmar surface of the hands and the skin of the face were particularly active. Prior cleansing of the skin with distilled water, ether or alcohol did not appear to affect the clot-promoting activity. When plasma samples from patients with congeni-

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