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Cell Culture Technics for Cytogenetic Investigation of Human Abortus Material

Analysis of 45 Cases and Report of 3 Specimens with Gross Chromosomal Aberrations

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AS A RESULT OF INVESTIGATIONS carried out in recent years, gross chromosomal aberration has been established as a major etiologic factor in early abortion.[19] One puzzling fact which has emerged from these cytogenetic investigations is that a considerable number of specimens examined have, for no apparent reason, failed to grow in culture. The proportion of failed cultures has varied in different series. Bové *et al.* and Carr, who have published results from the two largest series of abortions examined cytogenetically, quote a failure rate of 30% and 42%, respectively. Carr states that the 149 specimens which failed to grow in culture did not differ from the 200 successfully cultured specimens in respect to the patient's previous obstetric history or gestational age. Two possible explanations for such growth failure must be considered: (1) that it is due primarily to abnormalities inherent in the specimens, and (2) that it may be the fault of technical factors.

In this study, an attempt has been made to ascertain to what extent the growth failure rate is influenced by tissue-culture technic. Three different tissue-culture technics were used in the cytogenetic investigation of 45 unselected cases classified as spontaneous abortion. The methods used and the results obtained are described.

Of the 20 specimens which were successfully cultured, three were found to have abnormal karyotypes; the findings are described.

MATERIAL AND METHODS

The material consisted of 45 specimens obtained from patients admitted to our hospital, whose conceptions were judged to have aborted spontaneously. In 22 cases, a fetus or embryo was present; of these, 4 were first-trimester embryos, and 18 were second-trimester fetuses. The remaining 23 specimens consisted of products of conception without the embryo, which were derived from first-trimester abortions. Tissues were collected in dry, sterile containers, stored at

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4°C., and were set up in tissue culture within 12 hr.

An attempt was made to free trophoblastic tissue, as far as possible, from blood and decidua.

Embryos and fetuses were examined for the presence of gross congenital abnormalities and to estimate the approximate age (attention being given to the crown-rump length and degree of differentiation). In older embryos and fetuses, portions of skin with underlying fascia were removed from the back and the upper and lower limbs for culture purposes. In younger embryos, portions of limb bud were used and, occasionally, yolk sac.

All tissues were washed immediately before culture in Hank's balanced salt solution containing penicillin and streptomycin (100 ml. of Hank's solution contained 5000 units of penicillin and 0.004 gm. of streptomycin).

Culture Technics

METHOD I: CULTIVATION OF TISSUE EXPLANTS IN AIR-TIGHT BOTTLES. The method described here is that which is currently used in this laboratory for the cultivation of adult human fibroblasts.

The tissue was divided into small portions approximately 3 mm. in diameter. The average number of explants taken from each specimen was eight. Explants were placed in 4-oz. medical flats; in the case of skin, the undersurface was placed against the glass surface. Five milliliters of Waymouth's complete medium with 20% calf serum was added. The bottles were placed in a slanted position so that half of the explant was in the medium. Explants were incubated at 37°C.

In the case of successfully cultured fetal tissues, at approximately 4–7 days, a primary outgrowth could be seen which consisted of small round cells (histiocytes) and fine spindle-shaped cells. The small round cells subsequently disappeared and were replaced by more typical fibroblasts. Between the ninth and fourteenth days, the primary outgrowth was trypsinized into the culture bottle. From then on, cultures were fed at 3-day intervals and subcultured when confluent. At the third or fourth subculture, there were sufficient cells for air-dried drop preparations. Before the seventh serial passage, cells were suspended in medium containing 10% glycerol and stored at —70°C.

METHOD II: MASS TRYPSINIZATION AND GROWTH ON COVERSLIPS. The method used was a modification of that described by Thiede.[16] Mass trypsinization was undertaken in six specimens of trophoblastic tissue. Tissues were exposed for ½ hr. to 0.25% solutions of trypsin and to the action of a magnetic stirrer at low speed. The cell suspension thus obtained was centrifuged (600 rpm for 10 min.), and the supernatant trypsin was removed. Cells were resuspended in Waymouth's complete medium containing 20% calf serum and seeded onto coverslips in Petri dishes. The Petri dishes were then placed in a 37°C incubator with circulating carbon dioxide; the carbon dioxide concentration was adjusted to ensure a pH of approximately 7.4 in the culture medium.

METHOD III: CULTIVATION OF TISSUE EXPLANTS ON COVERSLIPS. This method is a modification of the technic described by Basrur *et al.* The material was divided into small explants approximately 2 mm. in diameter. Explants were then sandwiched between pairs of coverslips in Petri dishes: three to four explants were placed between each pair. Two milliliters of Waymouth's complete medium with 20% calf serum was gently pipetted into each Petri dish, and these were incubated in a 37° incubator with circulating carbon dioxide.

Sex Chromatin Studies

SEX CHROMATIN IN FETAL MEMBRANES. Where available, amnion or yolk sac were used for sex determination. The method employed was similar to that described by

Klinger and Schwarzacher. The membrane was stretched out on a cleaned glass slide and then fixed in a solution consisting of equal parts of ether and 95% ethanol. It was found that membranes could be stored satisfactorily in this solution for several weeks. A 1% solution of aqueous cresyl violet was used for staining. Typical sex chromatin could be demonstrated in about 50% of the cells in female controls and was absent in male controls.

SEX CHROMATIN IN CELLS IN CULTURE. Satisfactory preparations for examination of sex chromatin were also obtained from cells in culture. A suspension of cultured cells was obtained by trypsinization; cells were then resuspended in fixative composed of acetic acid:ethanol, 1:3, without hypotonic pretreatment, and drop preparations were made. These preparations were stained using cresyl violet as above.

Preparation of Cells for Chromosomal Analysis

AIR-DRIED DROP PREPARATIONS. This technic, a modification of that described by Moorhead *et al.*, was used where tissue had been cultured according to *Method I.* Thirty-six hours after subculturing, colcemid (1 ml. of a 80 μg./ml. solution) was added to the culture medium, and cells were incubated for a further 8 hr. Cells were then trypsinized off the surface of the culture bottles, with care being taken to avoid prolonged exposure to the trypsin solution. When the cells were free, usually after a 5-min. incubation at 37°C., tissue-culture medium was added to inhibit further action of the trypsin. The cell suspension was then centrifuged (600 rpm for 8 min.), after which the cells were resuspended in hypotonic solution (1.12% sodium citrate) and incubated in a 37°C. water bath for 7 min. The hypotonic solution was removed after centrifugation (600 rpm for 8 min.). Cells were then resuspended as evenly as possible in freshly prepared cold fixative, consisting of a 1:4 solution of acetic acid and alcohol. This cell suspension was refrigerated for 1 hr., after which the cells were again centrifuged and the resuspended in a small quantity of fresh cold fixative.

Drop preparations were made from a height of about 18 in. onto cold, wet slides and were dried gently over a flame. Preparations were stained in 2% natural orcein and then dehydrated and mounted. At least 20 cells from each specimen were counted and analyzed, and of these, two or more were photographed for karyotyping.

COVERSLIP PREPARATIONS. This technic is a modification of that of Tjio and Puck.

When outgrowth was considered sufficient, explants were transferred to fresh coverslips, and growth of the primary culture was allowed to continue for a further period of 48 hr. Colcemid (0.25 ml of a 80 μg./ml. solution) was added to the culture medium, and cells were incubated for 4 hr. The medium was then removed, and the coverslips were placed in Petri dishes containing hypotonic solution and incubated at 37°C. for 12 min. Coverslips were then exposed to fixative acetic acid and alcohol, 1:3. It was found advantageous to expose the coverslips to the vapors of the fixative for 1–2 min. before placing them in the solution. Preparations were treated with an aceto-orcein stain as above. The number of cells analyzed in specimens where the coverslip method was used varied between 5 and 10.

RESULTS

Culture Technics (Table 1)

CULTURE METHOD I. This method proved particularly suitable for growth of fetal or embryonic tissue (skin and underlying fascia, limb bud). A total of 22 specimens were cultured in this way; growth occurred in 18. Of the four specimens which failed to grow, three were badly macerated. Suitable preparations for chromosome studies were obtained from 16 of these specimens, which

TABLE 1. RESULT OF TISSUE-CULTURE TECHNICS

Tissue material	*Method*	*Specimens (No.)* Total	*Showing growth*	*Showing no growth*	*Providing suitable mitotic figures*
Fetal or embryonic	I. Growth in stoppered bottles	22	18	4	16
Trophoblastic	I. Growth in stoppered bottles; explants	8	5	3	1
Trophoblastic	II. Mass trypsinization; growth on coverslips	6	4	2	0
Trophoblastic	III. Growth on coverslips using explants	10	9	1	4

included 3 first-trimester and 13 second-trimester abortions. In two of them—one first-trimester embryo and 1 second-trimester fetus—initial cellular outgrowth was followed by regression of growth before cell numbers were sufficient for chromosomal analysis.

Method I proved less successful in the case of chorion and amnion. Eight specimens of these tissues were cultured in this way. In five specimens, an initial outgrowth of round, polygonal, and fine spindle-shaped cells was observed, usually after an interval of 3–5 weeks. In four of these specimens, the cells of the primary outgrowth did not survive trypsinization. In one specimen of trophoblastic tissue, the epitheloid and spindle-shaped cells were replaced by more typical fibroblasts, and growth was subsequently sufficient for drop preparations.

CULTURE METHOD II. In four of the six specimens treated in this way, confluent growth was observed after 2–3 weeks. This growth consisted mainly of polygonal and syncytial cell types. Suitable mitotic figures, however, could not be obtained from these specimens.

CULTURE METHOD III. This method would appear to be most suitable for cultivation of trophoblastic tissue for the purpose of chromosome analysis. Ten specimens were treated in this way; growth was noted in nine, and cells suitable for chromosomal analysis were found in four specimens. Using this method, outgrowth sufficient for making preparations for chromosome analysis was usually noted at 3–5 weeks and consisted of epitheloid and fibroblastic elements.

Karyotype Studies

Of the 22 specimens of fetal or embryonic material received, 16 (72%) were karyotyped. At least 20 cells were counted and analyzed in each specimen, and of these cells, two or more were photographed and karyotyped. In three of these specimens, numerical chromosome aberrations were detected. These specimens constituted three of the four first-trimester embryos received during the course of this study. In the remaining 13 specimens, no numerical or structural chromosome abnormalities were detected, although one specimen was noted to show heteromorphism of Chromosome Pair 16 (Fig. 1). This is a well-recognized normal variation which was also found in the mother, and thus is unlikely to have been responsible for the abortion.

Cells suitable for chromosome analysis were obtained from only 5 (20%) of the 24

Fig. 1. Karyotype analysis in embryonic fibroblast cell from abortus specimen showing heteromorphism of Chromosome Pair 16, a normal variation. (Aceto-orcein, reduced from × 4000)

specimens of trophoblastic tissue cultured. The number of cells analyzed in these specimens varied between 5 and 10. Of the five specimens karyotyped, four revealed no gross chromosome abnormalities. The remaining specimen proved to have a triploid constitution. In this triploid conception, both trophoblastic and embryonic tissues yielded cells suitable for chromosome analysis.

Description of Karyotypically Abnormal Specimens

CASE 1

Gross Morphology. This specimen consisted of an intact gestational sac, 10 cm. in diameter, containing a large amount of fluid and a very small embryo, approximately 1.5 cm. in total length, with few signs of differentiation beyond folding. Menstrual history suggested a gestational age of 8 weeks. A globular structure contained in the gestational sac was considered to represent the yolk sac. The embryo was divided into three portions, each of which was set up in culture as an explant. Yolk sac and amnion were used to determine sex chromatin.

Growth. Growth appeared to be slower than is usual for fetal material. The first subculture was carried out 4 weeks after initiation of the culture. Cell numbers were sufficient to allow for the making of air-dried drop preparations at the third passage. Cell growth, however, could not be maintained to provide sufficient numbers for storage purposes.

Chromosome Analysis. Forty cells were counted and analyzed; 39 cells were found to contain 45 chromosomes, and in 1 cell 44 chromosomes were present. Karyotype analysis suggested a 45/XO chromosomal constitution (Fig. 2); this was confirmed by sex-chromatin studies; no sex-chromatin body could be detected in cells of the amnion or yolk sac.

Family History. The father, aged 43 years, had two normal children by a previous mar-

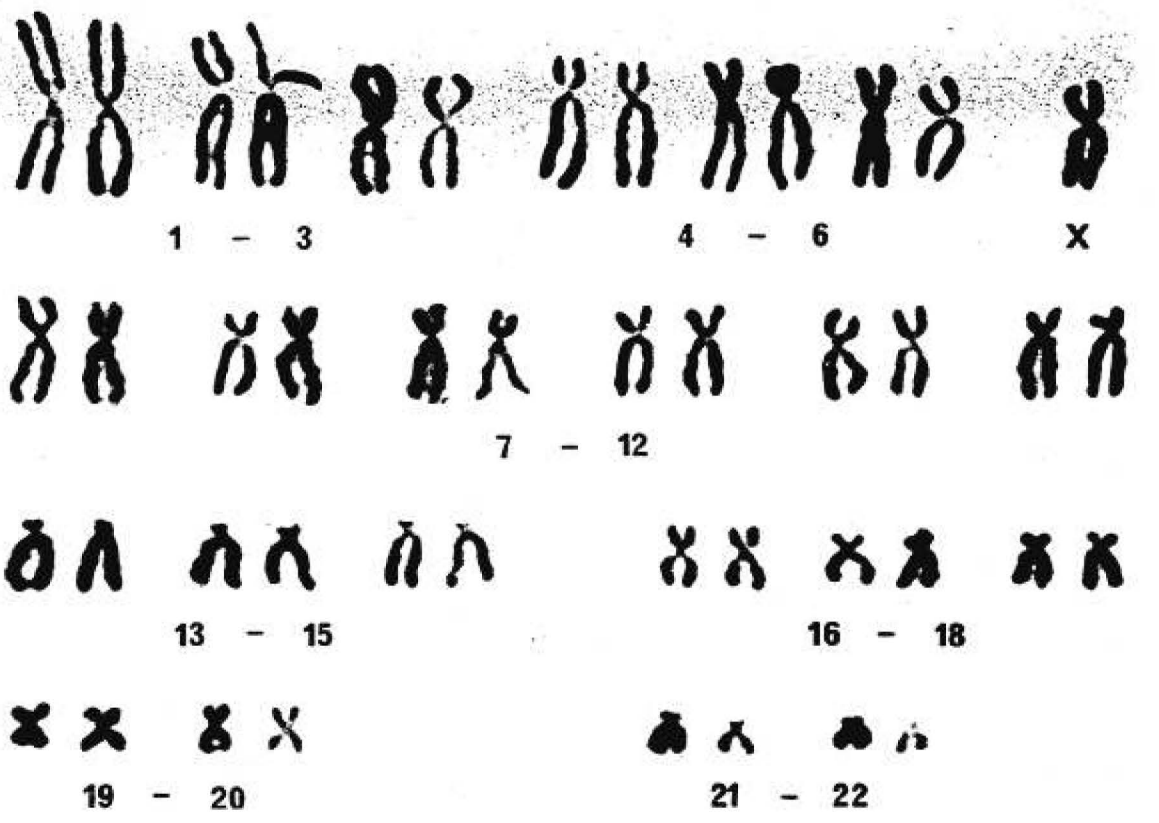

Fig. 2. Karyotype analysis of mitotic cell from embryonic fibroblast culture in Case 1, showing 44 autosomes and a single X chromosome. (Aceto-orcein, reduced from × 4000)

riage. The mother, aged 30 years, gave no history of previous miscarriages or of infertility.

Chromosome studies on lymphocyte cultures from both parents revealed no structural or numerical abnormalities.

CASE 2

Gross Morphology. This embryo showed differentiation compatible with a gestational age of 8 weeks. Measurements were not obtained owing to destruction in the cranial regions. Menstrual history suggested a gestational age of 8–9 weeks. Portions of the limb buds were used as explants. Cell outgrowth was first trypsinized at 2½ weeks. Growth appeared to be slower than is usual for fetal tissue, and cultures could not be maintained long enough to provide the volume of cells suitable for storage.

Chromosome Analysis. A total of 35 cells were examined; 18 of these cells had 45 chromosomes, while 46 chromosomes were present in 17 cells. Karyotype analysis suggested XO/XX mosaicism (Fig. 3 A and B).

Sex-Chromatin Studies. A total of 100 cells derived from fibroblast culture were examined, but no typical Barr body could be detected. This embryo was considered to be a 45/46 XO/XX mosaic. Decidual contamination giving rise to an XX line was not considered likely, as material for culture was obtained from embryonic limb buds. Furthermore, decidual tissue seldom grows vigorously under the culture conditions used here.

Family History. The father was aged 28 years, and the mother 20 years. There were no siblings, and no history of previous miscarriages or of infertility. Chromosome studies on lymphocyte cultures from both parents revealed no abnormalities.

CASE 3

Gross Morphology. This specimen consisted of an intact gestational sac, 15 cm. in diameter, containing a large amount of fluid and an embryo with crown-rump length 2.5 cm., suggesting a gestational age of 9 weeks. Menstrual his-

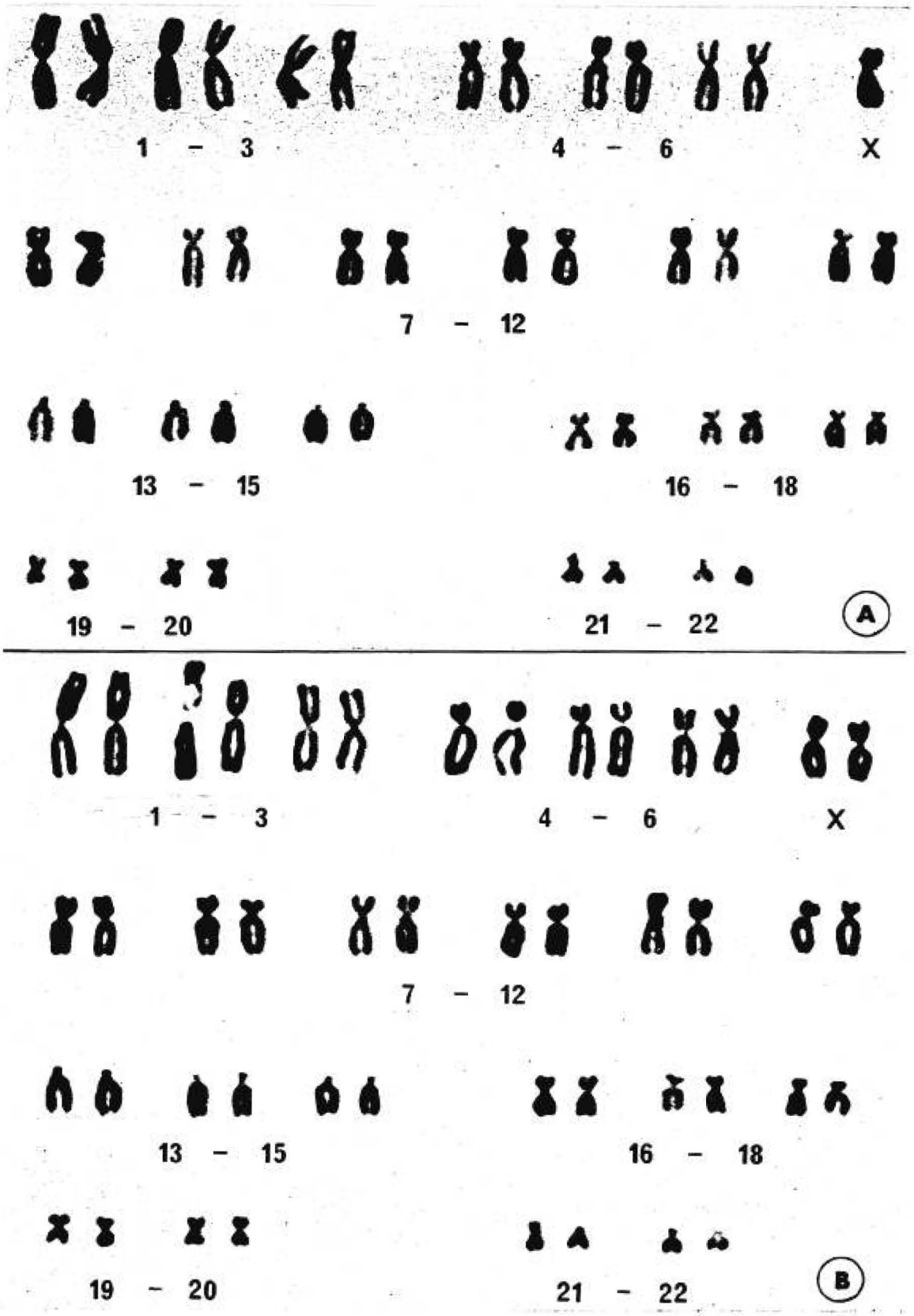

Fig. 3. Case 2. A. Karyotype analysis of 45/X cell from embryonic fibroblast culture. B Karyotype analysis of 46/XX cell from embryonic fibroblast culture. (Aceto-orcein, reduced from × 4000)

tory suggested a gestational age of 18 weeks. Cranial differentiation seemed to be incomplete, a fairly marked prominence being present in the cervical region. The lumber region of the spinal canal appeared to be abnormal, fusion of the posterior wall of the canal being incomplete. Tissue was removed from the limb buds for purposes of culture. Portions of the trophoblast were set up as explants on coverslips (*Method III*). The amnion lining the gestational sac was used for sex-chromatin examination; sex-chromatin bodies were found in 51 (17%) of 300 cells examined.

Growth of Chorionic Tissue. Outgrowth was considered sufficient for chromosome analysis after 21 days. Twelve cells proved suitable for examination; of these, 5 were found to have 69 chromosomes, and 7 had chromosome numbers varying between 65 and 68.

Growth of Embryonic Tissue. This compared favorably with that observed in normal embryos. Cell growth provided sufficient volumes for storage at the fifth and seventh passages. Drop preparations for chromosome analysis were made at the third trypsinization. Cells derived from fibroblast cultures showed a modal chromosome number of 69. Twenty cells were examined; 15 had 69 chromosomes; 2 cells were found to contain 70 chromosomes, while 3 cells showed counts of 67 and 68. Karyotype analysis revealed an XXY sex chromosomal complement (Fig. 4).

Family History. The mother and father were aged 30 years. No other siblings were reported. Also, there was no history of previous miscarriages. Chromosome analyses on lymphocyte cultures from both parents revealed no abnormalities.

DISCUSSION

Culture of Trophoblastic Tissue for Cytogenetic Investigation

A total of 24 specimens of trophoblastic tissue were cultured using the three technics described. Growth was noted in 16 specimens (66%), but only 5 (20%), yielded suitable mitotic figures for chromosome analysis. It is difficult to compare these results with those obtained in other similar studies, because in many such studies the percentage of specimens of trophoblastic tissue which yielded suitable metaphase plates is not quoted.

From our results, it would appear that maintenance of cellular growth from trophoblastic tissue presents a major problem—early regression of cellular outgrowth being

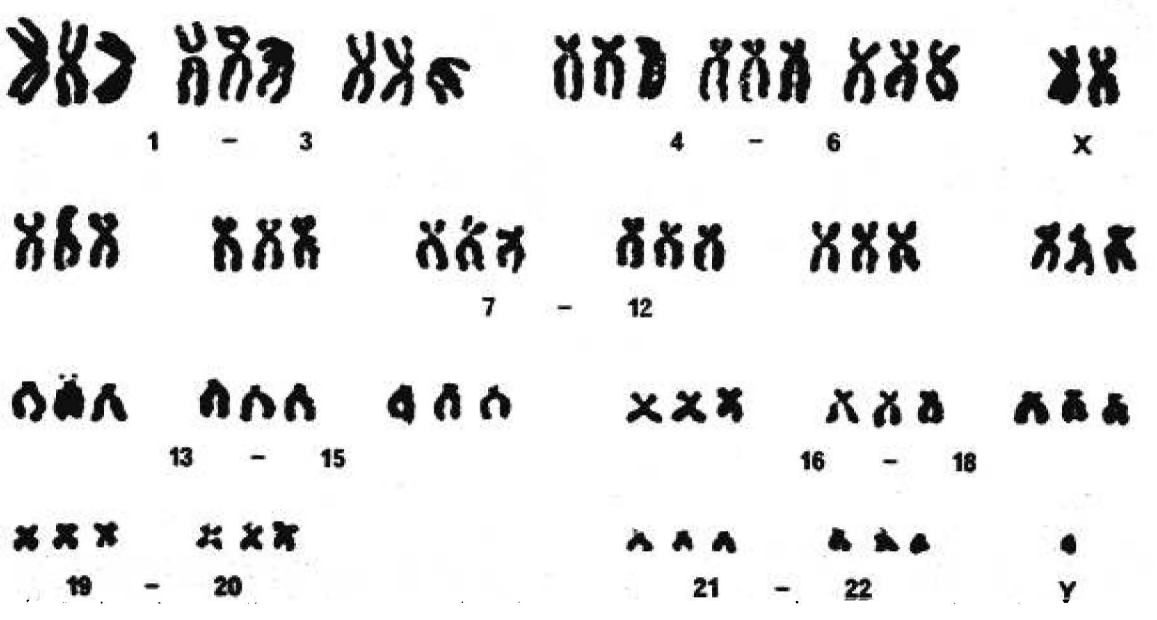

Fig. 4. Case 3. Karyotype analysis of 69/XXY (triploid) cell from embryonic fibroblast culture. (Aceto-orcein, reduced from × 4000)

a frequent feature of trophoblastic tissue culture. Thiede,[16] in his extensive study of the human trophoblast in tissue culture, noted that cultures were maintained only when a more resistant fibroblastic line replaced the epitheloid elements. Soma noted early regression of syncytial and polygonal cells derived from trophoblast. A method which facilitates use of the primary outgrowth from trophoblastic tissue for the preparation of metaphase plates, therefore, seems indicated. This would explain the failure of *Method I* in the case of trophoblastic tissue.

The fact that suitable mitotic figures frequently could not be found, despite good cellular growth on coverslips, proved most disappointing. This failure appeared to be due to a low mitotic index. Nakanishi noted very low divisional activity in cells obtained from amnion. In this study, divisional activity appeared to be particularly low following trypsinization, but rather higher during the stage of primary outgrowth from an explant. Therefore, to obtain suitable numbers of metaphase plates, large numbers of explants of trophoblastic tissue should be set up in culture, and the primary coverslip preparation should be examined early—e.g., at approximately 3 weeks.

Factors accounting for the difficulties in cultivation of trophoblastic tissue could be numerous. Possibly, mediums are required which are more suitable for cultivation of epithelial elements; most mediums in current usage select strongly for fibroblastic elements. In the few specimens studied here, growth appeared to be more satisfactory when tissue was cultured in Petri dishes in an atmosphere with circulating carbon dioxide. Possibly, the oxygen and carbon dioxide tensions existing under these conditions are particularly suitable for growth and metabolism of trophoblastic tissue.

One of the most interesting problems arising when trophoblastic tissue is cultured is that of possible contamination with decidual cells. Indeed, it is surprising that so few instances of XX/XY mosaicism in trophoblast cultures have been described. Thiede and Metcalfe[17] found one such example in material from 54 of the 179 spontaneous abortions cultured by them. Possibly, decidual cells grow less easily than do trophoblast cells under the usual conditions of culture.

Culture of Embryonic or Fetal Material for Cytogenetic Investigation

Where suitable material is available—i.e., fetal or embryonic tissue—the first method described here has numerous advantages, one of the most important of which is that many cells can be obtained without involving a high number of serial passages. Considerable volumes of cells are required for making drop preparations, which provide the most satisfactory mitotic figures. Furthermore, using this method, cell volumes suitable for storage can be obtained relatively early—in our experience usually before the seventh passage.

In order to obtain satisfactory preparations for chromosome analysis, it is important that a high percentage of cells in culture should be actively dividing at the time of harvesting. It was found necessary to draw a growth curve for fetal cells; from this curve it was established that the most active period of division occurred between 36 and 48 hr. after subculture. Therefore, colchicine was added to cell cultures during this period.

Comment on Cytogenetically Abnormal Specimens

In this study, karyotype analysis was undertaken at the end of the fourth passage of fetal cell cultures, when growth was proliferative. Hayflick and Moorhead, using human fetal cell cultures, noted that the diploid pattern remained intact during the interval when vigorous multiplication of cells took place. Only in the later stages, when degenerative changes appeared in cultured cells, could aneuploidy be demonstrated.[13] Chromosome abnormalities arising in vitro, therefore, would not be expected in our material.

XO Chromosome Constitution in Abortus Specimens

Carr has reported the most common single chromosomal abnormality encountered in abortus specimens to be a 45 XO chromosome constitution. This occurred in 12 of the 50 chromosomally anomalous specimens examined by him. On the basis of this finding and the incidence of spontaneous abortion, Carr has estimated that only 1 in 40 XO zygotes survives to term. It is indeed surprising that an abnormality which proves lethal in such a high percentage of zygotes should be compatible with survival in some cases.

Mosaicism in Abortus Material

Accumulated data published by the WHO Group in 1966 on 153 karyotypically abnormal abortuses showed that mosaicism (group unspecified) occurred in 12 specimens. Monosomy, trisomy, and triploidy all occurred more frequently. For mosaicism to be present in a significant number of cells in an individual, nondisjunction must occur during the early divisions of the zygote. Polani has suggested that mammalian placentation provides some degree of protection against mosaicism, since by far the greater volume of the blastocyst is involved in the formation of the trophoblast.

In the abortus described here, monosomy for a sex chromosome proved lethal, despite the presence of mosaicism for an apparently normal cell line.

Triploidy in Abortus Material

Carr found triploidy to be the second most common anomaly in abortus specimens, occurring in 9 of the 50 chromosomally abnormal specimens. In 2 triploid abortuses and in 1 diploid triploid mosaic abortus Schlegel *et al.* reported hydropic degeneration of chorionic villi similar to that described by Makino; 3 cases of triploidy described by Edwards failed to show this abnormality. Hydropic degeneration could not be detected on low-power microscopic examination of the chorion in the triploid abortus described in the present report. The abnormality of the spinal canal in our triploid embryo would appear similar to that found in a triploid embryo described by Edwards.

SEX CHROMATIN AND TRIPLOIDY. Sex chromatin findings in triploid individuals are of interest in relation to hypotheses concerning the mechanism of sex-chromosome inactivation. Sex-chromatin bodies were present in 17% of amnion cells examined in the XXY triploid described here, suggesting that X inactivation does occur in the presence of a balanced sex chromosomal-autosomal complement.

Edwards examined the amnion of an XXX triploid embryo; he reported a higher than normal incidence of Barr bodies and the occurrence of double-sex chromatin bodies in 4% of cells. Klinger and Schwarzacher found 10% of cells in normal amnion to be polyploid (tetraploid). Thus, the presence of double-sex chromatin bodies in amnion cells from an XXX triploid would be significant only when it could be established whether the particular cells containing these double chromatin bodies were triploid or hexaploid. This would seem possible on the basis of measurement of nuclear volume.

IN-VITRO GROWTH OF TRIPLOID CELLS. In this study, cell growth appeared to be less abnormal in the presence of triploidy than in the presence of sex chromosomal anomaly. One of the many explanations which could be implicated is that of cell volume. Harris has suggested that the slow progressive decline during serial culture might be due to leakage of enzyme protein, RNA, or other cell components which are not replaced at suitable rates under in-vitro conditions. He furthermore suggested that the selective advantage of polyploid cells in long-term culture could be explained on the basis of their more favorable surface-volume relationships. Hauschka showed that cell volume is a func-

tion of ploidy. Schlegel *et al.* found triploid cells to have a greater volume than diploid cells.

CONCLUSIONS

From the results of this limited study, we concluded that in order to obtain satisfactory metaphases plates from trophoblastic tissue, a large number of explants of this tissue should be set up in culture and that the primary outgrowth should be used for preparation of these plates.

Furthermore, it would seem advisable that in the reports of such investigations, results obtained with fetal or embryonic material and those from trophoblastic material should be reported separately. In this way, the optimal culture technic for each tissue type might be determined.

SUMMARY

During the course of a cytogenetic investigation of abortus material, it was noted that the high growth-failure rate was due primarily to difficulties in maintaining cellular growth from trophoblastic tissue. In order to overcome this problem, in the case of trophoblastic tissue, culture methods were employed which facilitated use of the primary outgrowth for the purpose of chromosome analysis. The tisssue culture methods used, and the results obtained therewith, are described.

In all, 20 of the 45 specimens received were successfully analyzed. Cytogenetic abnormalities were detected in three specimens; these specimens are described, and the karyotype and sex-chromatin findings in each are discussed. Chromosome analyses on peripheral blood obtained from the parents of these three abortions, revealed no numerical or structural abnormalities.

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