Technical methods

The selection of a method for the culture of cells from amniotic fluid

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Amniotic Fluid

There are many cells in amniotic fluid but the majority of them are dead squames from the foetal epithelium. The viable cells may come from various tissues—epithelial cells from the urinary tract or the respiratory tract, amniotic cells, or fibroblasts.

At 12 weeks of pregnancy there is between 8 and 85 ml of amniotic fluid (Wagner and Fuchs, 1962). At 17 weeks the average amount of fluid is 225 ml. The fluid consists of 98 to 99% water with 1 to 2% solids, of which the inorganic part resembles those of extracellular fluid. The remaining solids are half protein and half other organic compounds.

Specimens of amniotic fluid with which to practise culture techniques are not easy to obtain. Fluid can be obtained at term in large amounts but this is contaminated with vernix and pieces of amnion. The ideal specimen is from a pregnancy of 15 weeks' duration, when a clinical specimen would be aspirated. Specimens taken at this period have been obtained from two sources: (1) when fluid was needed for the estimation of Rh antibodies and (2) when a late termination of pregnancy was done, either by hysterotomy or by the introduction of an abortifacient substance into the uterine cavity. The amount of fluid to spare from the former source is usually small but in the latter case up to 20 ml was usually easily obtained. Specimens vary greatly in the number of cells present. On average, more cells were present at 15 to 16 weeks of pregnancy than at 12-13 weeks. Some fluid contained fresh blood. A small amount of blood seemed to improve the growth potential. Two specimens were dark brown and contained altered blood from a previous haemorrhage. This did not affect the growth of the cells.

Culture Vessels

The first vessels tried were Carrel flasks with silicone

rubber bungs which were very good when new but were difficult to clean. If the flask was scratched during cleaning it was often difficult to see the first few growing cells. This difficulty was also experienced with Leighton tubes. Plastic petri dishes specially prepared for tissue culture are made in a variety of sizes. Using dishes of 30 and 50 mm diameter experiments were done with various concentrations of cells in fixed amounts of fluid but neither the variation in size of the dish nor concentration of cells had any effect on cell growth. Other authors have experimented in similar fashion (Nelson and Emery, 1973). Cells can also be made to grow in a petri dish under a coverslip (Valenti and Kehaty, 1969). Growth in these vessels was no better than in the dish by itself.

The petri dishes were not of the locking type. During culture therefore they had to be in a CO₂ incubator with controlled humidity or in a sealed container within a standard incubator. Several containers were tried. The use of the petri dishes was finally abandoned because of the frequency of fungus infections. Infection was cut down by improved techniques and the use of fungicidal agents in the medium but was not abolished.

The culture vessel then adopted and still used was the plastic Falcon flask. Similar flasks are now produced by other manufacturers. The growing area is a little large (25 sq cm) to scan in the early stages of growth. They are however easy to use and can be gassed and sealed and therefore do not need a CO₂ incubator or container. The risk of contamination when the culture is changed is also lessened.

Culture Media

Most of the media in the various culture techniques described in the literature use between 15 and 30% of foetal calf serum, made up with one or other of the commercially available solutions such as 199, Ham's medium, or Eagle Hank (Jacobson and Barter, 1967; Nadler and Gerbie, 1970). Foetal calf serum is very expensive and varies widely in its ability to support growth. It has been suggested that when a good batch is discovered the remainder of the batch should be purchased (Nelson and Emery, 1973) but this would be difficult for a small unit since it would present storage problems and would be very expensive. A short time ago one large supplier of the serum was refused import licences

because of virus diseases in the cattle of the country of origin. Since almost all the serum is imported this could well happen again. Our attempts to grow cells in amniotic fluid failed and this was also the experience of other authors (Gray, Davidson, and Cohen, 1971).

Pooled human AB serum is now used since there seems to be little variation in its ability to support growth. The basic medium used is that originated by the Paediatric Research Unit at Guy's Hospital, London, with various additions, in an effort to improve cell adhesion and growth. These included small amounts of foetal calf serum, glucose phosphate broth, and chick embryo extract. The first two were abandoned as having no effect. The chick embryo extract appeared to give some improvement, but it is difficult to be certain of this because of the lack of strictly controlled experiments. A small batch of human embryo extract was made using material derived from three terminations of pregnancy by the vacuum extraction method. It was prepared by the method of Paul (1970). The extract was used in the cultures at various concentrations but did not appear to have any advantage over the chick embryo extract.

Apparatus

Shortly after the change to Falcon flasks a laminated flow bench was purchased. All media were prepared on this bench and all cultures put up and changed on it. These changes reduced the number of infections in subsequent cultures to nil. Our incubator is fitted with a thermistor temperature control and also a thermostatic override. Cells will easily withstand a night at room temperature but will not suffer overheating for very long. Our centrifuges are fitted with variable resistance rheostats to permit low rates of spinning. All our medium was sterilized by filtration using Sartorius and Millipore filters.

Culture Method for Amniotic Fluid Cells for Chromosome Analysis

MEDIUM

This was made up of 100 ml Biocult 199 with Hanks salts and Hepes, 20-30 ml human AB serum, 0.4 ml

chicken embryo extract (Biocult), 0.6 ml L-glutamine (Biocult), 60 µg/ml Crystapen, 100 µg/ml streptomycin.

Samples are usually put up for culture as soon as possible after the fluid is taken. If it is necessary to keep the sample for more than a few hours it is stored in a domestic refrigerator at 4°C. Successful growth has been achieved from samples stored for up to two days either at 4°C or at 36.5°C. Specimens are collected and stored in plastic sterilin bottles, first because they are coned ready for spinning, and secondly because cells do not adhere to the vessel while the specimen is in transit.

METHOD

Spin sterilin bottle containing 10-20 ml amniotic fluid at 800 rpm for 10 min. Pipette off supernatant and add a few millilitres of medium to pellet. Divide the cell suspension equally between two or four flasks depending on the density of cells. Make up volume of medium to 3 ml in each. Gas flask with 5% CO₂ in air. Incubate at 36.5°C for 5 to 7 days. Examine each flask for cell colonies. By this stage growth should be apparent. Remove medium and add 3 ml fresh medium. Continue to change medium every other day until cell growth is sufficient for harvesting. This may vary between nine and 14 days. Inject cultures with 0.1 ml of 0.02% Colcemid solution. Leave for four and a half hours. Trypsinize cells from surface with warmed 5% trypsin solution, diluted 1:1 with phosphate-buffered saline. Transfer cells and trypsin solution into siliconized centrifuge tube, using a siliconized pipette. Spin for 10 min at 700 rpm. Discard supernatant and add 5 ml 0.6% KCL solution, warmed to room temperature, for 13 minutes. Spin at 700 rpm for 10 minutes. Discard supernatant and fix with 3:1 absolute alcohol: glacial acetic acid. Spin at 1000 rpm and refix. Keep at -20°C for 45 min before spinning and putting cells on slides. The slides should be cleaned until completely grease-free, then stood in cold running water for approximately 30 minutes. If a water film has not formed over the entire surface the slide should be discarded. Great care must be taken in harvesting these cells to prevent loss of valuable divisions. When cells have been put on slides, dry gently in air and stain.

Experimental	Total No. Samples	No. Growing after One Week	No. Harvested	No. Karyotyped	No. Infected	Death from Unknown Causes	No. Failing in Harvest	Excessive Temperature	Total No. Failures
Period	127	79 (62%)	44 (34.6%)	33 (26%)	54 (42.5%)	28 (22%)	11 (8.5%)	1 (0.7%)	94 (74%)
Present method	31	31 (100%)	31 (100%)	27 (87%)			4 (13%)		4 (13%)
Total	158	110	75	60	54	28	15	1	98

Table Results in experimental period and using present method

RESULTS

The table shows that since the introduction of our present culture method all 31 specimens received have been successfully cultured. Four were lost in the harvesting and we believe we have now improved our technique. The number of successful cultures is now 60 and in this sample we have found three chromosome abnormalities.

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References

- Gray, C., Davidson, R. G., and Cohen, M. M. (1971). A simplified technique for the culture of amniotic fluid cells. *J. Pediatr.*, **79**, 119-122.
- Jacobson, C. B., and Barter, R. H. (1967). Intrauterine diagnosis and management of genetic defects. *Amer. J. Obstet. Gynec.*, **99**, 796-807.
- Nadler, H. L., and Gerbie, A. B. (1970). Role of amniocentesis in the intrauterine detection of genetic disorders. *New Engl. J. Med.*, **282**, 596-599.
- Nelson, M. M., and Emery, A. E. H. (1973). Amniotic fluid cell cultures. *J. med. Genet.*, **10**, 19-22.
- Paul J. (1970). *Cell and Tissue Culture*, 4th ed. Livingstone, Edinburgh and London.
- Steele, M. W., and Breg, W. R., Jr. (1966). Chromosome analysis of human amniotic fluid cells. *Lancet*, **1**, 383-385.
- Valenti, C., and Kehaty, T. (1969). Culture of cells obtained by amniocentesis. *J. Lab. clin. Med.*, **73**, 355-358.
- Wagner, G., and Fuchs, F. (1962). The volume of amniotic fluid in the first half of human pregnancy. *J. Obstet. Gynaec. Brit. Cwllth*, **69**, 131-136.

Screening of vasectomy specimens

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It is the usual practice following the operation of male sterilization by vasectomy to submit the excised length of vas deferens for histological confirmation of its structure. With the increasing popularity of the operation the number of specimens is forming a real burden to many histology laboratories. In the last year in Plymouth, specimens were examined from 590 patients, forming 7% of the total histopathology requests.

It is customary in most laboratories to embed and section each vas and to treat them in exactly the same way as other specimens to be subjected to histopathological investigation. The extra work load for pathologists is minimal, and of the 1180 specimens examined in 1972 in this laboratory all were confirmed to be vas deferens and no pathological changes were seen in any. The work load falling upon the technical staff, however, is considerable and an effort has therefore been made to find a quick screening test.

Methods

Specimens are received in buffered neutral formalin and stored and examined in batches.

Each vas deferens is divided transversely with a razor blade and as thin a slice as possible cut by hand with the razor blade. The resulting slice is held in a pair of watch-makers forceps and dipped for a few seconds in aqueous methylene blue, washed briefly in distilled water, then in 70% alcohol, followed by a further quick wash in distilled water. It is then mounted on a slide under a cover slip in Apathy's medium.

Results

By this very simple technique the vas can easily be recognized (see fig). The longitudinal folds in the mucosa, and the very thick intermediate circular muscle layer are quite characteristic. In cases of doubt, material can be embedded and sectioned in the usual way. Control specimens of arteries and veins of similar size have been examined and there is no difficulty in distinguishing these from the vas deferens.

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