hypertension or the borderline of pulmonary oedema from mitral stenosis. Unfortunately, however, this still occurs, and, in spite of the added difficulties of the diagnosis in pregnancy due to the changes in blood volume and anaemia, we feel surgery is indicated without delay if the possibility of pulmonary oedema is suggested by paroxysmal dyspnoea, repeated haemoptyses, basal rales, and radiological evidence of pulmonary congestion or hypertension.

We thank Dr. J. Johnston, consultant anaesthetist; Dr. J. W. Brown, head of the Sheffield Regional Cardiovascular Centre; and the members of the departments of cardiology, medicine, and radiology at the City General Hospital, Sheffield.

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PREPARATION OF CELL CULTURES FROM HUMAN AMNION

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It is more than two years since Zitcer and her associates (1955) showed that cell cultures derived from human amnions could be used to grow the poliomyelitis viruses. Since then amnion cell cultures have also been shown to be sensitive to a wide variety of other viruses, including members of the A.P.C. and Cocksackie groups (Weinstein *et al.*, 1956), ECHO viruses (Lahelle, 1957), and measles (Ruckle, 1957). The cultures are therefore especially suitable for use in the routine diagnosis of human infections, and, since little has been published in this country on the cultivation of this type of cell, we thought that a note of our experiences with them might be of value to others.

In the Biological Standards Control Laboratory amnion cell cultures have been used for poliomyelitis vaccine safety testing and for experimental work with these and other viruses. Although several different methods of preparing cultures have been tried, only the one that has been found most satisfactory and is in current use is described in full.

Primary Cell Cultures

Amnions from normal deliveries are obtained under as sterile conditions as can be procured in a busy maternity home without unduly inconveniencing the staff. Before delivery the patient is swabbed with a dilute hypochlorite solution and the midwife rinses her gloved hands in biniodide solution before transferring the placenta and membranes to a sterile dish. She replaces her gloves with a dry sterile pair before stripping the amnion manually from the placenta and chorion. The amnion is then transferred to a 250-ml. ground-glass stoppered bottle with a wide neck, which is sent to hospital the day before the amnion is required.

These bottles, containing phosphate buffered saline (P.B.S.) (Dulbecco and Vogt, 1954), 200 units of penicillin and streptomycin, and 25 units of nystatin per ml., are left at room temperature and are usually returned within 16 hours of delivery.

On receipt in the laboratory the membrane is transferred to a Petri dish and a piece of it taken for bacterial culture. After removal of clots, it is transferred to a 500-ml. Erlenmeyer flask and washed three times in Hanks solution. It is then covered with 250 ml. of medium 199 containing 5% human serum, 0.16% sodium bicarbonate, and antibiotics, and the flask is tightly stoppered. The amnion is left overnight at room temperature. If the broth shows no sign of bacterial growth next morning the amnion is washed three times in Hanks solution, and is then ready for trypsinizing. 250 ml. of P.B.S. solution containing 0.25% trypsin (Difco trypsin 1/250) is added and held at 37° C. for half an hour. The bottle having been gently swirled, the fluid containing red cells and debris is discarded. A further 250 ml. of trypsin is added and the bottle placed in a water-bath at 37° C. for three to five hours, depending on how many cells are required. After this time the bottle is swirled more vigorously and the fluid poured off into 250-ml. centrifuge bottles. After spinning for 10 minutes at 1,000 r.p.m., the supernatant is removed and the cells are washed once with Hanks solution. They are then re-suspended in 50 ml. of 199 solution containing 0.06% sodium bicarbonate and 20% pooled human serum. The cells are counted without staining and the final suspension is adjusted with this medium so that 1 ml. contains 300,000 cells. Bottles or tube cultures can then be initiated, using either soft or hard glassware. Pyrex feeding-bottles are seeded with 10 ml., 500 ml., medical flats with 40 ml., and test-tubes with 1 ml. of this suspension. Escro rubber stoppers or liners for the screw caps have proved satisfactory for sealing the cultures. Confluent cultures result after one to two weeks' incubation at 37° C.

Secondary Cultures

Usually second passage cells are used for making tube cultures. To prepare them from bottle cultures the cell sheets are washed three times in Hanks solution and covered with 0.02% "versene" in P.B.S. (solution A only), and the bottles held at 37° C. for 30 minutes. An equal quantity of 0.25% trypsin solution is then added and the bottles incubated for a further 15–20 minutes. The cells are removed from the versene-trypsin mixture by centrifugation, washed with Hanks solution, and re-suspended in medium 199 containing 20% human serum. Secondary cultures are ready for use after three to five days' incubation if an inoculum of 100,000 cells is used.

Maintenance of Cultures

For keeping cultures prior to use, medium 199 with 5–10% human serum and 0.16% sodium bicarbonate is satisfactory, the medium being replaced when its pH falls below 7. If the cells are kept at 31° C. the medium can be replaced less frequently.

When required for virus work the cells are maintained in medium 199 containing 0.14% bovine albumin, but if it is necessary to hold the cultures for more than five to seven days 2% horse or monkey serum is added.

Discussion

The method described above has been modified from that suggested by Weinstein *et al.* (1956), as the temperature of trypsinization has been raised from "room temperature" to 37° C. No adverse effect of 0.25% trypsin on amnion cells has been found at this higher temperature, as has been noted by Lahelle (1956), and the preparation time is shortened considerably. No increase in cell yield appeared to occur with repeated extractions (Zitcer *et al.*, 1955) or with vigorous shaking. The yields obtained seem to be of the

same order as those obtained by these workers. After three hours' trypsinization, 300 ml. of cell suspension has been regularly obtained, while after five hours this has been increased by about 50%. If these cells are first seeded in bottles they will yield from 500 to 750 tubes per membrane on subculture.

The time elapsing between delivery and receipt of the amnion in the laboratory does not seem to affect the subsequent growth of cells; specimens which have been received after an interval of up to 16 hours yield satisfactory cultures. Keeping the amnion for a further 24 hours in order to see whether it contains micro-organisms has resulted in no contamination of cell cultures processed since this step was introduced. The initiation of cultures does not appear to be affected by the antiseptics used during delivery. It is doubtful whether these ever come into contact with the amnion, as it is protected by the chorion and placenta and is itself handled only after a change of gloves.

Since the method described has been in use 40 amnions have been received, of which seven were contaminated. Of the 33 processed 30 gave good yields, while the other three grew poorly but on trypsinization yielded from 100 to 200 usable tubes. This degree of bacterial contamination was much the same as that encountered in a preliminary series of cultures, in which different methods of preparation were tried (10 out of 44). Between these two series a run of contamination was encountered which was due to a change of staff at the maternity hospital. It seems that the antibiotics used can take care of most of the micro-organisms that are bound to be present in all amnions not delivered by caesarean section.

Of the 72 amnions processed, 7 gave only a poor primary growth and 14 did not grow. All the latter were in the first series. Pooled human serum has been preferred to horse or calf serum, as these latter have given variable results, depending on the batch used, and have never produced such good cultures when tested in parallel with human serum. With human serum it is necessary to wash the cells at least three times to remove antibody before adding the maintenance medium if human viruses are to be studied. However, if racks are used such as those described by Syverton and Scherer (1954) or those used in our laboratory (see photograph), 200 tubes can be rinsed and the medium replaced in less than half an hour. The advantages of human over other sera outweigh this slight inconvenience.

For secondary cultures a combination of versene and trypsin (Fogh and Lund, 1957) has given a better yield than either alone.

Among the methods of trypsinization tried which gave yields comparable to those currently obtained were (a) three one-hourly extractions at 37° C. (Dr. Elsa M. Zitcer; personal communication), and (b) 0.25% trypsin at 30° C. for five hours (Labelle, 1956). Overnight treatment with 0.05% trypsin solution at room temperature gave growth but low yields (two experiments) and at 31° C. a better yield but poor growth (one experiment). With 0.1% trypsin solution overnight the yield was good but growth very poor (four experiments).

As amnion cultures do not maintain well in the absence of serum, their use in poliomyelitis vaccine testing is limited (Medical Research Council, 1957). They can be regularly maintained in bottles with 199 solution containing 0.14% bovine albumin for 10 days, but are usually beginning to degenerate by 14 days. However, they are still as sensitive as fresh monkey kidney cultures to the three types of poliomyelitis virus as long as this degeneration is not too advanced (see Table). With medium 199 containing 10% of a variety of sera, cultures can be maintained for many weeks in good condition with infrequent medium replacements, especially if incubated at 31° C.

Tubes can be rinsed without removing them from the rack, as they are held in position by detachable stainless steel springs. Square holes are used to ensure that the tubes locate positively in parallel positions and to provide a ready means of anchoring the springs to the rack. Larger racks holding 72 and 201 tubes are also in general use. The rack was designed by Mr. W. C. Lister and Mr. A. T. Davey, of the Engineering Division of the National Institute for Medical Research, London, N.W.7.

Besides the poliomyelitis viruses, we have found that B virus and pseudo-rabies will grow in these cells with cytopathic changes similar to herpes virus (Weinstein *et al.*, 1956) and that lymphocytic choriomeningitis virus multiplies readily, but without any visible cellular change.

As would be expected, normal amnion cells are much more resistant to x rays than cells in continuous culture (Puck and Marcus, 1956) and support the growth of poliomyelitis virus after large doses of radiation.

Although four or five successive subcultures have been made on several occasions, no continuous line of amnion cells has yet been isolated, as has been done by Fogh and Lund (1957).

Summary

The method of preparing amnion cell cultures used in the Biological Standards Control Laboratory is described

Comparative Titrations of Poliomyelitis Virus in Amnion and Other Cell Cultures

Time from Initiation of Culture to Inoculation	Time Incubated Without Serum before Inoculation	Primary (P) or Secondary (S)	Type 1 (Brunenders)			Type 2 (MEF-1)			Type 3 (Saukett)		
			Virus Titre in Amnion Cells	Virus Titre in Fresh Monkey Kidney Cell Cultures	Virus Titre in Fresh HeLa Cell Cultures	Virus Titre in Amnion Cells	Virus Titre in Fresh Monkey Kidney Cell Cultures	Virus Titre in Fresh HeLa Cell Cultures	Virus Titre in Amnion Cells	Virus Titre in Fresh Monkey Kidney Cell Cultures	Virus Titre in Fresh HeLa Cell Cultures
1 week	0	S (P2)*	7.8	7.6	7.8	6.2	6.1	—	5.8	5.4	—
2 weeks	0	P	7.1	7.1	—	—	—	—	—	—	—
4 1/2 weeks	2 weeks	P	7.0	6.5	—	6.0	5.6	—	6.0	5.6	—
2 "	3 "	P	5.8	5.8	—	6.0	6.25	—	5.6	5.3	—
5 "	3 "	P	6.5	6.0	—	6.1	5.7	—	4.9	5.0	—
4 days	3 "	S (P12)	6.5	6.5	—	5.7	5.5	—	4.9	5.5	—
6 "	0 "	S (P2)	7.4	7.6	6.9	6.0	7.6	6.3	6.1	6.5	5.6
6 "	0 "	S (P2)	7.1			6.3			6.5		
6 "	0 "	S (P2)	7.0			6.6			6.2		
6 "	0 "	S (P2)	7.7			6.8			6.8		

* Denotes age in weeks of primary cultures from which the secondary ones were made.

and is briefly compared with methods already reported. It is not claimed to be superior to others, but has been found to need little attention, is not too long, and is generally successful.

Thanks are due to the Sister Tutor and staff of the Queen Mary's Maternity Hospital, London, N.W.3, for their willing co-operation in supplying amnions regularly for this work; to Mr. F. C. R. Picton, of the Edgware General Hospital, Edgware, Middlesex, for some specimens from cases of caesarean section; and to Mrs. Angela Harris for technical assistance.

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Medical Memoranda

Clinical Trial of New Dressing for Varicose Ulcers

The very multiplicity of powders, lotions, and ointments which have been advocated for the treatment of varicose ulcers suggests that these ulcers generally heal in spite of, rather than because of, the local medication. A trial was made to find a simple and comfortable method of treatment for the ambulant patient, based on current knowledge of wound healing.

In the enthusiasm which followed the advent of the antibiotics it appears to have been largely forgotten that a granulating surface is itself an efficient antibacterial barrier. Inevitably such a surface will be contaminated and there will be a small amount of seropurulent discharge; nevertheless, healing proceeds apace. The ulcer may be infected, in which event the discharge will be frankly purulent, the granulations may be destroyed almost as rapidly as they are formed, and the ulcer may enlarge by the destruction of surrounding epithelium. In such an infected ulcer the damage is caused by the proteolytic enzymes liberated in the pus, and removal of the discharge as it forms will result in the infection being rapidly overcome by the body's own defences, and in healing. Mobilization of the body defences to the site of infection requires an adequate supply of good-quality blood, and attention must be directed to securing this by correcting anaemia and faulty diet, and by supporting the incompetent veins. Failure to improve the blood supply—and this may be impossible in some cases—results in failure to heal.

The principles of ulcer treatment, then, are attention to the blood supply, the provision of free drainage, and the avoidance of all trauma to the granulation tissue and migrating epithelium.

The "N-A" non-adhering dressing used in these trials is of a warp-knit filament rayon. Optimum conditions of "filament" character, mesh size, and fabric pattern have been determined by careful clinical experimentation. The completely smooth surface of the rayon filaments results in such a degree of non-adherence that the dressing is readily removed without any disturbance of new tissue. The fabric construction permits of free drainage, even of purulent exudates, and maceration of the surrounding skin due to inadequate ventilation is entirely avoided. Granulation buds, however, do not penetrate the mesh, and fibres of the absorbent backing are effectively kept from the ulcer surface. These factors together allow of infrequent change of dressings, and thus contribute towards faster healing. In the very rare cases where local medication may be thought

desirable, this may safely be used without fear of impairment of the above-mentioned properties.

METHOD OF TREATMENT

Without preliminary medication of any kind, the ulcer is covered with a single layer of N-A dressing. Owing to its non-adherent properties, it is usually necessary to hold the dressing in place whilst applying a backing of layers of absorbent gauze. The latter should be used in sufficient quantity to take up all discharge expected in the coming week. Absorbent gauze is found to be superior to cotton-wool and cellulose wadding as a backing medium; the last two afford insufficient ventilation and cause maceration of the tissues. Finally, the veins are supported by firmly bandaging the limb from the base of the toes to the knee with a porous elastic adhesive bandage. In order further to reduce venous stagnation the patient is instructed to walk about normally, to stand as little as possible, and, whenever sitting, to elevate the leg and to keep the toes moving. The patient returns for a change of dressing one week later, when it will be found that oedema is lessened or absent and the absorbent gauze will be soaked with exudate. The mesh of the N-A dressing, however, will still be open, the ulcer clean, and the surrounding skin quite dry. The material usually comes away with the gauze, but occasionally is lifted off with forceps. In neither case does it cause pain, discomfort, bleeding, or other tissue damage. A new dressing is then applied in the same way. As the ulcer heals and the amount of discharge lessens, the interval between dressings may be extended to two or even three weeks.

Experience has shown that some points must be emphasized. First, in those patients who are truly ambulant the ulcers heal and the discharge becomes minimal more rapidly than in those who are less active, which is in accord with the principle of improving the blood flow by use of the calf-muscle pump. The importance of walking must be explained to these patients, many of whom have for years been so consistently urged to rest the leg that they are almost afraid to use it. Those who by reason of infirmity are unable to walk benefit from instruction in toe and ankle exercise.

Secondly, patients who have become accustomed to daily dressings are sometimes loath to allow the leg to be bandaged for a week (several refused this form of treatment until they saw the rapid improvement of their fellow patients) and may take off the dressing unless warned that it may feel tight during the first week.

Thirdly, during the first one or two weeks of treatment of very extensive ulcers, underestimation of the amount of absorbent gauze required may result in the elastic bandage becoming stained with discharge. Whilst the dressing may safely be left for the full week, such profuse discharge causes discomfort and smell which it is kind to relieve by more frequent change of dressing.

RESULTS

The N-A dressing has been used for 250 dressings of 26 ulcers. In none of these has it become adherent to the ulcer, and on no occasion was there any bleeding or discomfort when it was removed. Examination of the removed dressing showed that the mesh of the material remained open in every case and that all discharge was freely transmitted to the overlying gauze. The ulcer itself was clean and required no attention beyond application of a new dressing. The surrounding skin was dry and healthy. Healing proceeded steadily, and the new epithelium was so good that it was usually difficult finally to identify the previously ulcerated area.

It is concluded, therefore, that N-A dressing is most satisfactory for the treatment of varicose ulcers. In avoidance of maceration, in reducing discomfort, and generally in promoting more rapid healing it is, in my experience, immeasurably superior to all dressings hitherto used for