Results

The three slides of the serial sectioned material—peroxidase uncounterstained, Perls' Prussian blue, and the peroxidase counterstained with Perls' Prussian blue—were carefully compared by matching the architecture of the fields using a comparison bridge. Neither the prolactin nor the haemosiderin components seemed to be diminished as a result of the extra staining procedure.

Low background staining resulted from the use of reasonably fresh potassium ferrocyanide crystals; the background staining appeared to increase in proportion to the increased shelf life of the crystals, although this was not measured histoquantitatively. But distinction could still be made between the peroxidase positive staining and the haemosiderin.

The increased time in washing after the osmium treatment, when performed with care, did not seem to increase the fragility of the sections, although more vigorous washing for longer periods was not attempted.

Discussion

Hormone identification plays an important part in the diagnosis of pituitary adenomas. Often, biopsy specimens obtained from surgically removed pituitary gland contain sites of old haemorrhage which are sparse, diffuse, and intermingled with the hormone producing cells. At the sites the differentiation between hormone and haemosiderin must be clear, which is why the technique was originally evaluated. It has previously been used successfully after the peroxidase-antiperoxidase method of Sternberger and Cucullis[4] in renal cell carcinoma,[5] where large amounts of iron pigment were present in the section.

With the increased use of immunoperoxidase techniques in surgical pathology,[6] the addition of this simple but old technique to the modern one could further increase its specificity.

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Storage of skin biopsies at −70°C for future fibroblast culture

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It is difficult to know when to establish fibroblastic cell cultures from paediatric necropsies. Newborn babies who seem to have defects due to chromosome aneuploidy may die before the results of lymohocytic karyotypes are known. Lysosomal storage diseases requiring confirmation by enzyme assay on cultured cells may be suspected only when microscopical examination of tissues has been completed. Establishing a culture from every necropsy is expensive. A simple method of storing a tissue sample for subsequent culture would be useful in these circumstances and would also have other practical uses in a clinical cytogenetics or tissue culture laboratory—for example, when samples are delivered at inconvenient times.

The cryoprotective effect of glycerol on frozen spermatozoa was reported in 1949.[1] This resulted in many techniques which describe the storage of frozen mammalian cells and tumours.[2–4] In 1959, dimethyl sulphoxide was used to prevent the haemolysis of frozen red blood cells.[5] Five years later Lehr *et al*[6] described the successful transplantation of skin autografts which had been previously frozen using cryoprotective agents.

We report here a simple method of storing skin biopsies (2–3 mm^3) at −70°C in culture medium plus dimethyl sulphoxide for 15–23 days without loss of capacity for fibroblastic cell growth.

Material and methods

Eight human skin biopsies, removed at necropsy, were used. Each was divided into several 2–3 mm^3 and 1 mm^3 samples on arrival in the laboratory. One of the 2–3 mm^3 samples was set up for culture immediately; the remaining samples were frozen for later culture.

Biopsies were transported from the postmortem room to the laboratory in 5 ml screw capped tubes containing 20 mM Hepes buffered Eagle's modified basal medium (Flow Laboratories) with 10% fetal calf serum (Flow Laboratories) plus 12·5 μg/ml neomycin sulphate (Hoechst-Roussel Pharmaceuticals), 12·5 μg/ml polymyxin B sulphate (Wellcome Foundation Ltd), 2·5 μg/ml fungizone (ER Squibb and Sons), and 10 μg/ml tylosin (Flow Laboratories).

Cultures were established by dicing the sample into small pieces with a scalpel and scratching the pieces into the dry base of a 50 × 13 mm vented petri dish, making sure each fragment was firmly attached before adding 7 ml of medium with 10% fetal calf serum plus antibiotics. The petri dishes were then placed in a 37°C, 5% CO_2, humidified incubator. Many techniques describing primary fibroblast culture have been previously reported.[7–10]

Samples for storage were placed at −70°C (Series 100 Kelvinator) in 5 ml tubes containing medium with 10% fetal calf serum plus antibiotics and 10% vol/vol dimethyl sulphoxide (Sigma Grade 1). Two additional 2–3 mm^3 samples (BT and LR) were frozen in fetal calf serum plus 5% vol/vol dimethyl sulphoxide and in medium without dimethyl sulphoxide. These were stored for the same length of time as the samples in medium plus 10% dimethyl sulphoxide.

Frozen biopsies were thawed in a 37°C room. When the ice had melted just enough to free the tissue, the icy medium and the biopsy were tipped into a petri dish. The biopsy was picked up using a scalpel, briefly rinsed by dipping in 5 ml of fresh medium, and set up for culture. At this stage a small amount (1 mm^3) of thawed tissue from one frozen sample (RC) was refrozen and stored for a further 14 days.

The petri dishes were examined and the medium replaced every seven days. When there was sufficient fibroblast outgrowth the cells were dispersed using phosphate buffered saline without Mg^{++} and Ca^{++} containing 0·025% wt/vol trypsin and 0·02% wt/vol edetic acid. As each petri dish reached confluency the cells were subcultured into a 25 cm^2 flask or 80 cm^2 flask (Nunc). The flasks were fed with medium containing 10% fetal calf serum plus polymyxin B sulphate or neomycin sulphate twice a week. The petri dishes with their initial epithelial outgrowth and original tissue still attached were refed and continued to produce more fibroblasts.

Results

Both the fresh and frozen 2–3 mm^3 size samples from the various biopsies grew at similar rates. Four of the seven frozen 1 mm^3 samples grew at rates comparable to their frozen 2–3 mm^3 counterparts. Two frozen 1 mm^3 samples (LR and JM) had no primary growth, however; these were also the slowest growing frozen 2–3 mm^3 samples. The 1 mm^3 RC sample, which was refrozen, reached confluency (25 cm^2 flask) after culturing for 37 days (Table).

After storage in fetal calf serum plus 5% dimethyl sulphoxide, BT and LR each produced a confluent 80 cm^2 flask after culturing for 28 and 33 days, respectively. When stored in medium without dimethyl sulphoxide, BT reached con-

Growth of fresh and frozen skin biopsies

Sample	*Growth of 2–3 mm^3 fresh skin*		*Growth of 2–3 mm^3 frozen skin*		*Growth of 1 mm^3 frozen skin*		*No of days frozen at −70°C*
	First day of fibroblast growth	*Day of confluent 80 cm^2 flask*	*First day of fibroblast growth*	*Day of confluent 80 cm^2 flask*	*First day of fibroblast growth*	*Day of confluent 80 cm^2 flask*	
BB	17	32 (25 cm^2 flask)	13	26	13	30	23
NW	15	26	13	26	13	26	23
RR	8	25	6	24	13	24	23
EH	8	25	12	20	12	23	17
RC	Not set up		5	24	20 (Refrozen biopsy)	37 (25 cm^2 flask)	15
LR	15	29	28	48	No growth after 62 days		16
JM	30	54	27	46	No growth after 47 days		17
BT	12	23	11	28	Not set up		15

fluency in 37 days (80 cm^2 flask) while LR had poor growth after 48 days in culture.

Discussion

This method allows the storage of a small skin biopsy from every necropsy in which there is the slightest possibility of a need for a fibroblastic cell culture, for long enough to determine whether culture is required or not. Later, cultures can be established if necessary or samples can be discarded when the need for a culture has been eliminated.

Further experience (data not presented) has shown that the skin biopsy can be left in the transport medium at room temperature for several hours before adding dimethyl sulphoxide and freezing. This procedure has also been used successfully for other tissues such as tumours and calf tendon.

We are conducting further experiments to determine how long biopsies can be kept at −70°C and whether transfer to liquid nitrogen after holding at −70°C overnight will allow long term preservation.

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Active site directed inhibitor used in the production of antibodies against urokinase

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Plasminogen activators are highly specific proteases found in trace quantities in most human tissues. These enzymes play important roles in tissue reorganisation and in the prevention of fibrin deposition. Urine contains a plasminogen activator, urokinase, which is not generally detected in normal tissues. Increased plasminogen activator synthesis is a feature of malignant transformation and may be specifically related to invasive and metastatic properties of tumour cells.[1 2] In a number of instances urokinase antigen has been detected as a malignant cell product, especially from ovarian tumours, and it has been suggested that radioimmune assay for urokinase might play a role in the detection of carcinoma.[3]

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The development of antibodies suitable for immune assay has been difficult owing to the lack of suitably purified plasminogen activators. This report describes a method by which antibodies to urokinase were obtained without prior purification of the antigen.

Material and methods

Glutamyl-glycyl-arginine-chloromethyl ketone (Glu-Gly-Arg-CH_2-Cl) was synthesised by Dr Elliott Shaw, Brookhaven National Laboratory, New York, USA, and kindly supplied by Dr D Pepper, Scottish National Blood Transfusion Service, Edinburgh, and Dr RAG Smith, Beecham Pharmaceuticals, Epsom, UK. This peptide inhibits