PRESERVATION OF HUMAN TRACHEAL ORGAN CULTURES AT -196°C

G. J. MORRIS*, R. BUCKLAND, D. RUBENSTEIN AND J. FARRANT

*Divisions of Cryobiology and Communicable Diseases,
Clinical Research Centre, Harrow, HA1 3UJ*

ALTHOUGH various tissue-culture cells have been preserved in liquid nitrogen at -196°C (Smith, 1961; Meryman, 1966; Mazur *et al.*, 1969) there have been no reports of the successful recovery of organ cultures for use in virology after freezing and thawing. The usefulness of organ cultures for growing viruses (Tyrrell and Blamire, 1967; Hoorn and Tyrrell, 1969) and for the investigation of anti-viral drugs (Herbst-Laier, 1970) is now well established. The survival of cells during freezing depends largely on the rate of cooling and on the concentration of cryoprotective additive (Rapatz and Luyet, 1965; Leibo *et al.*, 1970; Morris and Farrant, 1972). From a study of these factors we have been able to develop a method of storing embryonic human trachea at -196°C so that, after thawing, it retains apparently normal ciliary activity and susceptibility to infection with influenza virus.

MATERIALS AND METHODS

Tissue. Tracheas were obtained from 14–24-wk human embryos and organ cultures were prepared and maintained in 50-mm plastic petri dishes by the technique of Hoorn and Tyrrell (1965). Two pieces of tissue were cultured in each dish in 2.5 ml of medium.

Freezing technique. Organ cultures were suspended in Eagle's basal medium (GIBCO) containing 2 per cent. foetal calf serum and various concentrations of dimethylsulphoxide (DMSO) or sucrose. Two explants were frozen in 1 ml of medium in an open polypropylene tube (Sterilin Ltd).

The method of varying the cooling rate was that of Morris and Farrant. Tubes were positioned in an aluminium holder equidistant from its centre, to minimise variability in their cooling rates. The holder was then placed in an alcohol bath maintained at 1°C below the known freezing point of the suspending medium, and after 5 min. each sample was seeded by touching the surface of the medium with the tip of a Pasteur pipette containing some of the same medium already frozen. After a further 5 min. the holder was transferred to the interior of a freezing vessel cooled in liquid nitrogen. The inner temperature of the vessel was arranged to be the same as the temperature of the sample at the moment of transfer. Various kinds of freezing vessel were used.

- (a) An evacuated, unsilvered Dewar (90 mm $\times$ 250 mm) containing 250 ml of industrial methylated spirits (IMS), continually stirred, with a cooling rate of 0.3°C per min.
- (b) An unevacuated, unsilvered Dewar (90 mm $\times$ 250 mm) containing 500 ml of IMS, continually stirred, with a cooling rate of 1.6°C per min.
- (c) An unevacuated, unsilvered Dewar (90 mm $\times$ 250 mm) containing 250 ml of IMS, continually stirred, with a cooling rate of 2.4°C per min.
- (d) An empty, evacuated, unsilvered Dewar (90 mm $\times$ 250 mm) with a cooling rate of 4.9°C per min.
- (e) An empty, unevacuated, unsilvered Dewar (90 mm $\times$ 250 mm) with a cooling rate of 7.5°C per min.

Cooling rates were calculated with copper constantan thermocouples (28 swg) attached to a Rikadenki potentiometric recorder, as the time taken for the sample temperature to fall

Received 1 June 1972; accepted 6 June 1972.

* Medical Research Council Scholar.

from -15°C to -60°C . All samples were plunged directly into liquid nitrogen when their temperatures had fallen to -60°C .

Thawing was carried out in a waterbath at 37°C , the tube being rapidly shaken until melting was complete.

Testing for explant viability. After freezing and thawing, the two explants from each tube were washed three times in Hanks' saline and cultured in a petri dish in Eagle's basal medium with 0.2 per cent. bovine plasma albumin (BPA) and $100\text{ }\mu\text{g}$ per ml each of penicillin and streptomycin. After incubation for 24 hr to confirm sustained ciliary activity, the thawed cultures and control cultures, that had been prepared at the same time but had not been frozen, were infected with 1000 EID₅₀ of influenza virus per dish. They were then incubated for 3 hr to allow virus to adsorb, washed three times to remove unadsorbed virus and re-incubated at 33°C . The cultures were inspected for ciliary activity daily for 14 days. The medium was harvested and replaced with fresh medium every 2 days for 7-9 days. The harvested fluids were mixed with an equal volume of nutrient broth and stored at -70°C until they could be titrated for virus infectivity in 10-day-old embryonated hen eggs.

TABLE I

Preservation of ciliary activity in human tracheal organ cultures after freezing at different cooling rates in 25 per cent. dimethylsulphoxide

Cooling rate ($^{\circ}\text{C}$ per min.)	Proportion of organ cultures showing ciliary activity after thawing
0.3	6/6
1.6	4/6
2.4	3/6
4.9	0/6
7.3	0/6

Virus. Influenza virus A₂/Eng/939/69 was isolated by Dr A. S. Beare and has been passaged many times in the allantoic cavity of 10-day-old embryonated hen eggs. Stock virus was stored, as infected allantoic fluid, at -70°C .

RESULTS

In preliminary experiments, tracheal organ explants were frozen in medium without or with 5, 10, 20, 25 or 30 per cent. DMSO (w/v) at a cooling rate of 0.3°C per min. Ciliary activity was recovered, after thawing, only with DMSO concentrations of 20 per cent. or more. In the actual presence of DMSO there was no ciliary activity, but after washing the explants to remove the DMSO the activity returned. Similar reversible inhibitory action of DMSO on ciliary activity was found in control, non-frozen cultures. In contrast, sucrose (20 per cent. w/v) had no inhibitory effect on the ciliary activity of control cultures. Table I shows that the recovery of ciliary activity in explants frozen in 25 per cent. DMSO was dependent on the rate of cooling. This was maximal with a rate of cooling of 0.3°C per min. and was progressively less with faster cooling rates. In a single experiment with 25 per cent. DMSO, in which serum was omitted from the suspending medium, there was no recovery of ciliary activity, even with a cooling rate of 0.3°C per min. When 20 per cent. sucrose was used instead of DMSO there was no return of ciliary activity after freezing at any of the cooling rates studied. For all subsequent experiments, therefore, organ cultures were frozen in Eagle's basal medium containing serum and 25 per cent. DMSO at a cooling rate of 0.3°C per min. It was found that seeding procedure during freezing could be omitted without affecting the results.

The number of explants showing ciliary activity was similar in the frozen-thawed and in the control, non-frozen cultures for up to 8 days, after which time activity decreased more rapidly in the frozen-thawed cultures (table II). After infection with influenza virus, both

TABLE II

Duration of ciliary activity in frozen-thawed and in control, non-frozen human tracheal organ cultures, uninfected and after infection with influenza virus

Type of culture	Infected (+) or uninfected (-)	Percentage ciliary activity* on day						
		0	1	2	4	6	8	14
Control	—	100	100	100	100	100	90	70
Frozen-thawed	—	100	100	100	100	100	60	20
Control	+	100	100	100	50	5	2.5	0
Frozen-thawed	+	100	100	100	10	2.5	0	0

* Estimated by visual observation of four explants.

FIGURE.—Growth of influenza virus in frozen-thawed (○) and in control, non-frozen (●) human embryonic tracheal organ cultures.

the frozen-thawed and the control cultures always showed greatest loss of ciliary activity at the same time. Moreover, both types of culture produced essentially the same amounts of virus over the 9-day period of observation (figure).

DISCUSSION

It is clear from recent work (Rapatz and Luyet, 1965; Leibo *et al.*, 1970, Morris and Farrant, 1972) that both the concentration of cryoprotective additive and the rate of cooling are important for the optimum preservation of cells during freezing and thawing. The present results show that maximal survival of human embryonic trachea, as judged by ciliary activity and growth of influenza virus, occurred with a higher concentration of DMSO (25 per cent.) and a slower cooling rate (0.3°C per min.) than has been reported for most other mammalian cell types. Cryoprotection appears to require the presence also of serum in the suspending medium.

The freezing procedure reported here makes practical the preservation of viable human tracheal organ cultures for experimental purposes and could probably also be used for the storage of other ciliated organ cultures. This is perhaps particularly important now that embryonic tissue is becoming less readily available.

SUMMARY

Human embryonic tracheal organ cultures have been frozen to -196°C and thawed without any apparent loss of ciliary activity or susceptibility to the growth of human influenza virus. Optimal preservation was obtained by freezing in the presence of 25 per cent. dimethylsulphoxide at a cooling rate of 0.3°C per min. This extremely simple technique facilitates the storage of tracheal organ cultures for experimental use.

We would like to thank Dr D. A. J. Tyrrell for his helpful advice.

REFERENCES

- HERBST-LAIER, R. 1970. Organ cultures in evaluation of antiviral drugs I. Virus infection in trachea explants as potential model systems for evaluation of antiviral drugs against respiratory viruses. *Arch. ges. Virusforsch.*, **30**, 379.
- HOORN, B., AND TYRRELL, D. A. J. 1965. On the growth of certain 'newer' respiratory viruses in organ cultures. *Br. J. Exp. Path.*, **46**, 109.
- HOORN, B., AND TYRRELL, D. A. J. 1969. Organ cultures in virology. *Progr. Med. Virol.*, **11**, 1.
- LEIBO, S. P., FARRANT, J., MAZUR, P., HANNA, M. G., JR, AND SMITH, L. H. 1970. Effects of freezing on marrow stem cell suspensions. Interactions of cooling and warming rates in the presence of PVP, sucrose, or glycerol. *Cryobiology*, **6**, 315.
- MAZUR, P., FARRANT, J., LEIBO, S. P., AND CHU, E. H. Y. 1969. Survival of hamster tissue culture cells after freezing and thawing. Interactions between protective solutes and cooling and warming rates. *Cryobiology*, **6**, 1.
- MERYMAN, H. T. (Editor) 1966. *Cryobiology*, New York.
- MORRIS, G. J., AND FARRANT, J. 1972. Interactions of cooling rate and protective additive on the survival of washed human erythrocytes frozen to -196°C . *Cryobiology*, **9**, 173.
- RAPATZ, G., AND LUYET, B. 1965. Effects of cooling rates on the preservation of erythrocytes in frozen blood containing various protective agents. *Biodynamica*, **9**, 333.
- SMITH, A. U. 1961. Biological effects of freezing and supercooling, *London*.
- TYRRELL, D. A. J., AND BLAMIRE, C. J. 1967. Improvements in a method of growing respiratory viruses in organ cultures. *Br. J. Exp. Path.*, **48**, 217.