NOTES

Viability and Infectivity of Measles Virus-Infected Nervous Tissue Frozen in Cryoprotective Medium

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Measles virus-infected hamster brain tissue remained viable when frozen slowly in medium containing dimethyl sulfoxide. This procedure was essential for isolation of neurotropic measles virus from tissue specimens stored frozen.

Attempts to recover measles virus from patients with subacute sclerosing panencephalitis, a chronic infection of the central nervous system (CNS; 1, 8), invariably failed when the tissue specimens used for isolation were previously stored at −20 C or −70 C. Conditions at which virus isolation became successful (9, 15) indicated that viable brain cells were required for transmitting CNS-grown measles virus into the cell culture system (2, 17). It was concluded that preservation of virus infectivity in brain tissue at subzero temperatures would be impossible unless conditions were selected to protect organized glia and nerve cells from freezing damage without impairing consecutive trypsinization and cultivation of the dispersed tissue.

The literature contains many reports on the effects of freezing on the survival of tissues, cells, and organisms (5, 13, 16). The only report dealing with nervous tissue describes preservation and recovery of functional activity in the cervical ganglion of the rat after cooling to −76 C (14).

The present investigation attempted to preserve the infectivity of CNS specimens under given circumstances, either when immediate processing was not practicable or when later reference to the original material might have been required.

The experimental model used in these studies was measles encephalitis produced in hamsters by an adapted neurotropic measles virus strain (3) described in detail elsewhere (17). In short, brains removed from animals with advanced symptoms of encephalitis were minced, suspended in a cryoprotective medium containing dimethyl sulfoxide (DMSO), and frozen at different cooling rates to −70 C. After storage for 1 to 2 days, the samples were quickly thawed by agitation in a 37 C water bath. After several washings to remove the DMSO, the tissue was dispersed by adding 50 volumes of 0.25% trypsin and was subsequently incubated at 37 C for 30 min. The cell harvest was diluted in an equal volume of Eagle's minimal essential medium supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), and streptomycin sulfate (100 µg/ml). Cell viability was evaluated by observing the rate of outgrowth in petri dishes; the capacity to transfer neurotropic measles virus into cell cultures was assayed by titration in Vero cell cultures (17).

In view of the reported toxicity of DMSO for several kinds of cells and tissues (4, 18), a preliminary experiment was performed in which infected minced hamster brain tissue was suspended in 7, 10, and 15% DMSO for a time and at temperatures commonly employed in the pre- and postfreezing periods of the cooling experiments. The results (Table 1) indicate that none of these exposures had an appreciably adverse effect on consecutive trypsinization, cell outgrowth, and virus recovery.

In the next experiment, the rate of temperature drop was measured in test samples exposed to various temperature differentials. Generally, a slow rate of cooling, on the order of 1 C/min, was considered most suitable for preservation of nucleated cells suspended in a cryoprotective medium (16). The cooling curve of a sample exposed to a cooling gradient of −1 C/min is shown in Fig. 1. Immersing the test samples in

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TABLE 1. Effect of dimethyl sulfoxide (DMSO) on the growth potential and cell-mediated virus infectivity of minced infected brain tissue

| Per cent of DMSO in medium | Temp | TCID<sub>50/0.1 ml</sub> | Brain cell outgrowth |
|---------------------------|------|--------------------------|---------------------|
| DMSO in medium<sup>a</sup> |      |                          |                     |
| 0<sup>b</sup>             | C    | 56<sup>c</sup>           | 70 to 80<sup>d</sup> |
| 7                         | 22   | 56                       | 70                  |
| 10                        | 23   | 100                      | 70                  |
| 15                        | 23   | 56                       | 40 to 50            |
| 7                         | 32   | 32                       | 75                  |
| 10                        | 4    | 32                       | 80                  |
| 15                        | 36   | 56                       | 70                  |

<sup>a</sup> Medium consisted of Eagle's minimum essential medium with 10% fetal bovine serum. Brain tissue was kept in all media for 30 min.
<sup>b</sup> Control.
<sup>c</sup> Antilogarithm of TCID<sub>50</sub> values.
<sup>d</sup> Per cent of the total area of the culture vessel covered after 7 days.

![Schematic diagram of cooling in a sample placed in a freezing unit at the controlled rate of cooling of 1 C/min](image)

These experiments in a dry ice-ethanol bath resulted in a temperature drop of 24 C/min without a distinguishable transition phase (curve IV, Fig. 2). Placing the samples in a mechanical freezing unit at −20 C, −40 C, or −70 C produced curves II to IV (Fig. 2), with cooling rates of 1 to 4.5 C/min in the prefreezing phase and slow cooling rates of .65 to 1.0 C/min in the postfreezing phase. The transition period, i.e. the interval between the freezing point and resumption of faster cooling, was protracted only in curve IV (2 C drop over 24 min).

The effect of the four cooling regimens on the survival of hamster brain tissue was examined in four consecutive experiments listed in Table 2. Freezing of infected brain tissue without any additive destroyed the viability of the brain cells as well as their capacity to transfer measles virus into Vero cells. Infectivity for suckling hamsters was not abolished, a phenomenon in keeping with previous experience, in which brain tissue disrupted by homogenization lost infectivity for cell cultures but still was infectious for suckling hamsters (17). In most instances in which brain tissue survived the freezing procedure, virus could be transferred into cell cultures. Freezing in 7% DMSO in a −20 C unit followed by transfer to −70 C (cooling curve III, Fig. 2) gave positive virus recovery in three consecutive experiments. Freezing at a rate of 30 C/min (cooling curve IV) was successful with 7% DMSO but not with 15% DMSO. A prolonged freezing plateau (curve IV) was detrimental for cell growth and prevented virus transfer completely.

It is generally accepted that the most injurious factors in freezing and thawing of nucleated cells are (i) the exposure of cells to growing ice crystals and increasing electrolyte concentration in the transition period, (ii) intracellular ice formation during the rapid postfreezing cooling rates, and (iii) eruptive recrystallization during the rewarming period (5, 16). Addition of DMSO is thought to protect cells from freezing damage by reducing intracellular and extracellular ice formation and, possibly, by having a direct stabilizing effect on the cellular membrane lipo-protein. The variables which influence survival of cells during the freeze-thaw cycle have been reviewed (5, 13, 16) and help to explain the experience that widely differing cooling regimens may be required for the optimal survival of different cell types (6, 10, 12).

Results reported here confirm the expected high susceptibility of nondissociated brain tissue to freeze-thaw damage. Best protection seemed to occur when 7% DMSO and slow cooling at a rate of 1 C/min were employed. Preliminary ex-

![Rate of cooling of brain tissue at temperature differentials existing in I, CO<sub>2</sub>-ethanol bath; II, III, and IV, mechanical freezers set at −20, −40, and −70 C as used in the reported experiments](image)
TABLE 2. Effect of cooling infected brain tissue to subzero temperatures on preservation of viability and infectivity

| Expt | Cooling curvea | Medium in which brain tissue was suspended during freezing | Infectivity of trypsinized brain cell suspension | Brain cell outgrowthb |
|------|----------------|-----------------------------------------------------------|-----------------------------------------------|----------------------|
|      |                |                                                            | TCID<sub>50</sub>/0.1 ml in Vero cells | LD<sub>50</sub>/0.1 ml in suckling hamsters |
|      |                |                                                            |                                               | %                   |
| 1    | I              | None                                                      | 0<sup>a</sup>                                | 250<sup>c</sup>  |
|      | III            | None                                                      | 0                                            | 288                 |
|      | III            | DMSO (7%)<sup>d</sup>                                      | 80                                           | 379                 | No growth |
| 2    | I              | None                                                      | 0                                            | 8                   | No growth |
|      | III            | None                                                      | 0                                            | 0                   | No growth |
|      | III            | DMSO (7%)                                                  | 126                                          | 158                 | 70       |
| 3    | I              | DMSO (7%)                                                 | 8                                            | 21                  | 50       |
|      | III            | DMSO (7%)                                                 | 5                                            | 7                   | 50       |
|      | IV             | DMSO (7%)                                                 | 0                                            | 0                   | No<sup>e</sup> growth |
| 4    | Unfrozen<sup>f</sup> | NA<sup>f</sup>                                       | 100                                          | ND                  | 95       |
|      | I              | DMSO (7%)                                                 | 30                                           | ND                  | 5        |
|      | II             | DMSO (7%)                                                 | 18                                           | ND                  | 40       |
|      | IV             | DMSO (7%)                                                 | 0                                            | ND                  | 40       |
|      | I              | DMSO (15%)                                                | 0                                            | ND                  | No growth |
|      | II             | DMSO (15%)                                                | 10                                           | ND                  | 20       |
|      | IV             | DMSO (15%)                                                | 0                                            | ND                  | 5        |

<sup>a</sup> See Fig. 2.
<sup>b</sup> Per cent of the total area of the culture vessel covered after 12 days.
<sup>c</sup> Antilogarithm of TCID<sub>50</sub> or LD<sub>50</sub> values. ND, not done.
<sup>d</sup> Seven per cent dimethyl sulfoxide in Eagle’s minimum essential medium with 10% fetal bovine serum.
<sup>e</sup> Sample of brain tissue trypsinized and titrated immediately after harvest (control for freeze-thaw damage).
<sup>f</sup> NA, not applicable.

Experience has shown that freezing of trypsinized brain cells instead of minced brain tissue gave equal or better virus recovery after thawing and titration of the brain cells in Vero cultures.

Our results indicate that, when latent or defective, or otherwise suppressed virus infections are suspected, representative tissue material can be stored frozen in a viable state, permitting later attempts to transfer the presumed agent into a suitable indicator system.

LITERATURE CITED

1. Adels, B. R., D. C. Gajdusek, C. J. Gibbs, Jr., P. Albrecht, and N. Rogers. 1968. Attempts to transmit subacute sclerosing panencephalitis and isolate a measles related agent, with a study of the immune response in patients and experimental animals. Neurology 18:30-51.

2. Burnstein, T., and D. P. Byington. 1968. On the isolation of measles virus from infected brain tissue. Neurology 18:162-164.

3. Burnstein, T., J. H. Jensen, and B. H. Waksman. 1964. The development of a neurotropic strain of measles virus in hamsters and mice. J. Infec. Dis. 114:265-272.

4. Chang, C-Y., and E. Simon. 1968. The effect of dimethyl sulfoxide (DMSO) on cellular systems. Proc. Soc. Exp. Biol. Med. 128:60-68.

5. Doebbler, G. 1966. Cryoprotective compounds. Review and discussion of structure and function. Cryobiology 3:2-11.

6. Greene, A. E., R. K. Silver, M. Krug, and L. L. Coriell. 1964. Preservation of cell cultures by freezing in liquid nitrogen vapor. Proc. Soc. Exp. Biol. Med. 114:462-467.

7. Greiff, D., and W. Rightzel. 1966. Freezing and freeze-drying of viruses, p. 697-728. In H. T. Merryman (ed.), Cryobiology. Academic Press Inc., New York.

8. Harter, D. H., and J. Tellez-Nagel. 1968. Attempts to isolate SSPE agent in cell culture. Neurology 18:133-137.

9. Horta-Barbosa, L., D. A. Fusillo, W. T. London, J. T. Jabbour, W. Zeman, and J. L. Sever. 1969. Isolation of measles virus from brain cell cultures of two patients with subacute sclerosing panencephalitis. Proc. Soc. Exp. Biol. Med. 132:272-277.

10. Lewis, J. P., M. Passovoy, S. A. Conti, P. A. McFate, and F. E. Trobaugh, Jr. 1967. The effect of cooling regimens on the transplantation potential of marrow. Transfusion 7:17-32.

11. Luyet, B., and L. Menz. 1967. On the recovery of leukocytes from frozen suspensions containing dimethyl sulfoxide. Cryobiology 3:380.

12. Mazur, P., J. Farrant, S. P. Leibo, and E. H. Y. Chu. 1969.
Survival of hamster tissue culture cells after freezing and thawing. Cryobiology 6:1–9.
13. Merryman, H. T. (ed.) 1966. Cryobiology. Academic Press Inc., New York.
14. Pascoe, J. E. 1957. The survival of the rat's superior cervical ganglion after cooling to −76°C. Proc. Roy. Soc. Ser. B 147:510–519.
15. Payne, F. E., J. V. Baublis, and H. H. Itabashi. 1969. Isolation of measles virus from cell cultures of brain from a patient with subacute sclerosing panencephalitis. New Engl. J. Med. 281:585–590.
16. Rowe, A. W. 1965. Biochemical aspects of cryoprotective agents in freezing and thawing. Cryobiology 3:12–18.
17. Schumacher, H. P., and P. Albrecht. 1970. Optimal conditions for isolation of a neurotropic measles virus from brain tissue. Proc. Soc. Exp. Biol. Med. 133:398–401.
18. Sherman, J. K. 1965. Pretreatment with protective substances as a factor in freeze-thaw survival. Cryobiology 1:298–300.