Viral Studies on Human Brain Culture Retrieved from Cold Storage

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Received for publication 21 February 1975

Tissue cultures established from brain tissues of six patients with various chronic, degenerative diseases did not show evidence of viral agents. Sucrose gradient analysis of radioactively labeled culture fluid from two patients failed to reveal the presence of any incomplete or defective virus. Extracellular fluids from three brain cultures contained a factor able to stimulate deoxyribonucleic acid synthesis of Vero cells.

Many chronic degenerative diseases of the central nervous system are suspected of having a viral etiology. Recent successes in viral isolation using cell fusion and cocultivation of brain tissues with susceptible cells may eventually result in the characterization of the causal agents. In particular, parainfluenza type 1 virus was isolated from the brains of two multiple sclerosis (MS) patients, measles-like virus from patients suffering from subacute sclerosing panencephalitis, and papovavirus (JC type) from patients with progressive multifocal encephalopathy (4, 6, 10, 13, 14, 16, 18, 19).

More recently, considerable attention has been given to chemical treatment of cultures of human and animal tissues suspected of harboring latent oncogenic viruses. For example, Epstein-Barr virus was activated in a previously virus-free Burkitt lymphoma cell line after treatment with bromodeoxyuridine (BUDR) (9). Various murine C-type viruses have been recovered from virus-negative lines treated with iododeoxyuridine (IUDR) as well as BUDR (1, 11).

This laboratory has been examining brain specimens from patients with chronic central nervous system diseases suspected of having a slow-virus etiology. The approach to date has been to screen a number of cultured brain specimens for the presence of viruses by using both standard and special viral isolation techniques. In an effort to intensify the search for viral agents, tissues from a small number of patients with unusual case histories from a collection in this laboratory were reexamined with several previously untried methods, namely treatment with BUDR, ultraviolet (UV) irradiation, and radioactive labeling of cell cultures. Two previously tried methods, cocultivation and IUDR treatment, were also included but were performed both singly and in combination with additional indicator cell lines. It was hoped that new information might be obtained by concentrating the effort on a smaller number of patients with an expanded repertory of techniques.

MATERIALS AND METHODS

Patients. MS 1, a 13.5-year-old girl, after suffering visual difficulties, nystagmus, ataxia, loss of function and sensation in the lower extremities, was diagnosed as a case of MS. She died 22 months after onset of symptoms. A more detailed case study on this patient was presented by Adams et al. (3).

MS 2, a 16-year-old boy, was diagnosed as a case of MS after a history of remission and exacerbations of visual loss, weakness of extremities, and loss of bowel and bladder control. He did not improve after treatment with oral prednisone, progressed into a coma, and then died after 2 years of illness.

ALS 1, a 68-year-old man, was hospitalized after a diagnosis of amyotrophic lateral sclerosis (ALS). He had a history of lymphocytic meningitis and multiple relapses over a period of 23 years. He died 18 months after onset of ALS. An extensive report on this patient has been given by Norris et al. (12). Although the original tissue culture studies were negative, possible viral material in the form of serpentine tubules was observed in the axons of the patient's brain (12).

ALS 2, an elderly woman, was diagnosed clinically as a case of amyotrophic lateral sclerosis. Previous studies indicated that cultures of brain tissue from this patient hemadsorbed monkey erythrocytes (RBC), but not baby chicken, adult chicken, guinea pig, human O, rhesus monkey, and sheep RBC (7).

Leu 1, female, age 4, died from staphylococcal pneumonia after an infection with varicella virus. She had lymphoblastic leukemia with central nervous system involvement.

ADD 1, a 16-year-old girl, died after 6 months of progressive mental deterioration characterized by myoclonic jerks. Diagnosis at death was atypical demyelinating disease (2).
ALS 3, a 53-year-old man, died from respiratory paralysis approximately one year after a diagnosis of ALS.

Rub 1 was a 24-year-old man with congenital rubella whose neurological state was stable during the first 10 years of life. During the second decade of life he developed subacute motor and mental deterioration and died at the age of 24.

ALS 1 and Leu 1 were of special interest because of the history of viral infection in the course of their illness. ALS 2 was included because of the hemadsorbing activity of cultures established from her brain tissue. MS 1, MS 2, and ADD 1 were selected because of the possible presence of more active lesions in view of their young age at the time of their death. ALS 3 and Rub 1 were added toward the end of the study to study additional cultures for deoxyribonucleic acid (DNA) stimulatory effect (see Materials and Methods).

**Tissue culture.** All the tissues were postmortem brain and muscle material explanted between 1970–1971 and established in culture, as described by Cremer et al. (7). The cultures were subsequently frozen in sealed ampules containing 10⁶ cells in 1 ml of fortified Eagle medium in Earle balanced salt solution supplemented with 10% fetal bovine medium (FEE) and 10% Me₂SO. The ampules were retrieved from liquid N₂ storage and the cell suspensions were thawed at 37 C. The suspending fluid containing Me₂SO was removed after centrifugation of the cell suspensions. The cell pellet was resuspended in 15 ml of FEE and incubated at 35 C in an atmosphere of 5% CO₂ in air. After the formation of a monolayer, the cells were subcultured at weekly or longer intervals depending on the growth rate of the cells.

**Cocultivation.** The following indicator cells were used: baby hamster kidney (BHK-0853), human embryonic (EHS), human fetal diploid lung, African green monkey (Vero), dog sarcoma (D17), and human rhabdomyosarcoma. BHK-0853 is a continuous, morphologically transformed line established in this laboratory. EHS was obtained from the Naval Biomedical Research Laboratories, Oakland. D17 was obtained from John L. Riggs and RD from Robert McAllister. The first four lines were selected for their susceptibility to various viruses causing human disease and the last two for their susceptibility to various C-type viruses. The brain cells were mixed with indicator cells at a ratio of 2:1 either at the time of planting or after the brain cells attached to the culture vessel.

**Chemical treatment.** Brain cultures were incubated with either IUdR or BUdR at concentrations of 15 μg/ml or 30 μg/ml. After 48 h the culture fluids containing the drugs were replaced by fresh FEE. After 5 days, the cells were dispersed with 0.2% trypsin mixed in an equal volume of 0.02% ethylenediaminetetraacetic acid, resuspended in fresh medium, allowed to attach to the vessel, and then cocultivated with each of the indicator cells mentioned above.

**UV irradiation.** Confluent cultures of the brain cells in petri dishes, after removal of growth medium, were irradiated for varying periods from 1 to 60 s by a germicidal lamp (2537 A wavelength, Westinghouse, 15 W) delivering 80 ergs/mm² per s at 25 cm from the dish. After UV treatment, fresh FEE was added to the dishes. The irradiated cells were periodically examined for cytopathic change (CPE).

**Detection of viruses.** The brain cultures and the mixed cultures were checked for the presence of virus by (i) CPE and (ii) hemadsorption, carried out as described previously by Cremer et al. (7). The following physical method was also used to detect the presence of suspected viruses. Growing, subconfluent brain cultures in 16-ounce (ca. 0.473 liter) prescription bottles were labeled with either [³H]uridine (2 μCi/ml, specific activity 29 Ci/mmol) or [³H]thymidine (2 μCi/ml, 20 Ci/mmol) for 3 to 7 days. Thirty milliliters of the culture fluid from each line was collected, clarified, and centrifuged for 1 h at 100,000 × g. The resulting pellets were each suspended in 0.5 ml of buffer (0.02 M tris(hydroxymethyl)aminomethane, pH 8.0, + 0.5 M KCl + 0.006 M β-mercaptoethanol + 20% glycerol) and centrifuged through 45% sucrose gradients in a Beckman SW39 rotor at 35,000 rpm for 2.5 h. From 25 to 28 fractions were collected from each gradient directly onto glass fiber filters previously saturated with 0.2 ml of 10% trichloroacetic acid. The filters were washed under suction with 5 ml of 5% cold trichloroacetic acid followed by a rinse with 95% ethanol at 0 C. The acid-precipitable radioactive material trapped on the filters was measured in a toluene-base scintillator.

**Stimulation of DNA synthesis and the labeling of cocultivated cultures.** A mixture of 10⁶ cells to be tested and 2 × 10⁶ Vero cells was planted in each well of microtiter plates in triplicate. Control wells contained a corresponding number of either test cells or Vero cells alone. At the indicated time, 4 μCi of [³H]thymidine per ml was added to the cells. After 18 h, the cells were washed, removed with a mixture of equal amount of 0.2% trypsin and 0.02% ethylenediaminetetraacetic acid, precipitated with 5% trichloroacetic acid, and filtered through fiber glass filters, and the acid-precipitable radioactivity was measured. When tissue culture fluid was tested in place of cells, 0.1 ml of extracellular fluid, filtered through a membrane filter (45-μm pore) (Millipore Corp.), was added to 2 × 10⁶ Vero cells per well. Control wells contained 0.1 ml of FEE in place of the culture fluid.

In studies on the stimulation of DNA synthesis, additional cultures established from tissues from different sources were included: thoracic cord and muscle from ALS 3; muscle from ALS 2; and five different portions of the brain from Rub 1. The following cell lines established from human tumors and supplied by John L. Riggs were also included: lymphoma H37, neuroblastoma H3, papilloma H134, and carcinomas H123 and H133. Tissues from patients ALS 3 and Rub 1 were supplied by Forbes Norris and J. R. Baringer, respectively.

**RESULTS**

**Brain cultures.** Cultures which had been stored at -170 C for up to 2 to 3 years were successfully reestablished. Provided that the initial culture was growing well at the time, that
it was frozen, and that Me₂SO was removed from the cell suspension after retrieval from cold storage, the frozen cells grew satisfactorily in culture within 7 to 14 days. The reestablished cultures, like the original cultures, were mostly fibroblastic in appearance. The only exception was the culture from ADD 1, which was a mixture of fibroblastic and epithelial cells. The reestablished cells were subcultured every 4 to 5 weeks, with weekly feeding, and could be maintained for ±15 passages if the original cultures were frozen within five subcultures of the initial explants.

As with the original cultures, those established from frozen cells showed no morphological nor cytopathic changes upon subculture. Hemadsorption assays using various species of RBC confirmed the previous finding. No brain lines, with the exception of ALS 2, showed hemadsorption, and this, as before, hemadsorbed only monkey RBC (7).

Chemical treatment. Of the two drugs used, BUdR was generally the more toxic to the brain cells at either of the two concentrations tried. IUdR was moderately well tolerated at a concentration of 15 µg/ml but not of 30 µg/ml. The treated cells showed progressively slower growth with subculturing, but with no obvious CPE. Chemically treated cells cocultivated with indicator cells were uniformly negative when checked for CPE.

UV irradiation. Irradiation resulted in the death of 90% or more of the original cells within 24 h. This was true with even 5 s of exposure. After 2 to 3 passages postirradiation, the cells ceased to form confluent layers and grew in patches. After some 9 months of weekly feeding and observation, no unusual morphological changes were observed in the irradiated cells.

Cocultivation. Mixed cultures of brain with indicator cells were subcultured weekly and examined during six consecutive passages for the presence of CPE. With one exception, no unusual observations were made with any of the mixed cultures. None of the mixed cultures hemadsorbed guinea pig or monkey RBC. The one exception was MS 1 brain cells mixed with Vero, where the latter failed to attach initially. Clones of the monkey cells, distinguishable by their epithelial morphology, were visible only after a prolonged lag of 3 to 5 days. This initial toxic effect of MS 1 cells on monkey cells was transient and could be observed only during the initial three passages after cocultivation. This phenomenon was consistently observed but difficult to quantitate. The toxicity was cell associated; cell-free fluids from MS 1 cultures had no effect on Vero cultures. The coculture during this toxic phase showed no hemadsorption with either monkey or guinea pig RBC.

[^H]thymidine incorporation of mixed cultures. Although it appeared that MS 1 brain cells inhibited initial attachment of Vero cells in a mixed culture, it was not known whether the failure to attach was also accompanied by inhibition of growth. To test this possibility, DNA synthesis or uptake of [^H]thymidine was measured in brain or Vero cultures alone and in mixed cultures of the two. If the brain or Vero cells exerted no influence on each other, the sum of the thymidine incorporated by the two individual cultures should equal that observed in the mixed culture. On the other hand, if they were either antagonistic or synergistic, the incorporation for the mixed cultures and sum of the individual cultures would not coincide.

Stimulation index (SI) is defined as the ratio of [^H]thymidine incorporated by mixed culture to the sum of radioactivity incorporated by Vero and test cells alone. If the brain cells have no effect on Vero cells, the SI expected is 1.0. If the effect is stimulatory or inhibitory, SI would be greater or less than 1.0, respectively.

The results from preliminary labeling experiments were unexpected. DNA synthesis in mixed MS 1 and Vero cultures did not decrease. Up to 5 days after mixing, total incorporation of [^H]thymidine in mixed cultures equaled the sum of incorporation of the two individual cultures alone. Between 6 to 8 days after mixing, Vero cells were slightly stimulated, rather than inhibited, by MS 1 brain cells. Under microscopic examination, epithelial cells, morphologically Vero cells, were present in greater numbers in the coculture than in the control culture, indicating a faster growth rate in the former.

To ascertain if this was a phenomenon common to cocultivated cells, a number of human cell lines from different tissues were also tested. Included were cultures of the brain tissue from the six patients in this study and cultures of muscle tissue from ALS 2 and ALS 3. Subsequently, cell-free culture fluid was used as well as cells from the various cultures in the assay to test whether the increased DNA synthesis could be due to a soluble factor in the extracellular fluid. In the latter series of experiments, culture fluids from several human tumors, from the brain culture of a congenital rubella patient, Rub 1, and from normal human fetal diploid lung were included. A summary of all the experiments is shown in Fig. 1.

The data show that the cells and fluids tested fell into two classes, namely, those with SI > 1.9 and those with ≤1.7. Analysis of 13 samples of
cells and fluids in the latter category (an average SI value was used if more than one experiment was performed on any one sample) showed that the mean SI was 1.06, with standard deviation of 0.42. A cut-off point was therefore assigned at SI of 1.8 to differentiate the two classes. All those samples with SI > 1.8 were considered positive, and all those ≤1.8, negative.

The results suggested the following. (i) The cells and tissue culture fluid tested produced either no response or stimulated the Vero cells. (ii) Only cultures of brain tissues or their culture fluid were stimulatory. Of the seven brain specimens, three (MS 1, ALS 2, Rub 1) fell into the stimulatory class. In the case of ALS 2, muscle tissue was available for testing but was found to have no effect on Vero cells. (iii) Although both cells and fluid from MS 1 and ALS 2 were stimulatory, cells and fluids from each patient stimulated Vero cells in varying degree. The significance of this difference is unknown at present.

Preliminary data on the stimulatory factor, using culture fluid from Rub 1 cultures, indicated that the activity resides in the soluble fraction of the culture fluid. The pellet fraction after sedimentation at 100,000 × g did not stimulate Vero cells. The activity in the fluid fraction was unstable to freezing and thawing. The activity was destroyed within a few weeks during storage at −20°C, even in the presence of 20% glycerol and 0.006 M β-mercaptoethanol.

**Sucrose gradients of culture fluids.** All the above mentioned viral detection techniques require biological integrity of the suspected virus. They would not detect incomplete or defective viruses. In an attempt to look for the presence of virus in an aberrant form, the brain cultures were labeled with [3H]uridine or thymidine alone. The collected fluid was concentrated 60 times by centrifugation at 100,000 × g and centrifuged through 15 to 45% sucrose gradients. If any incomplete or defective virus was present, there should occur one or more discrete peaks with expected density between 1.10 to 1.50 g/ml. This range would include all but extremely fragmented viruses. A marker virus, rat C-type, was prepared from a [3H]uridine-labeled, chronically infected rat thymus line, in parallel with the brain cultures. Culture fluids from MS 2 and ADD 1 were examined this way. Both were negative. Very little radioactivity was detected beyond density of 1.06 g/ml on the gradients, regardless of the label used, whereas the marker virus banded at the expected density of 1.14 g/ml. Not enough cells from the other patients were available to test in this manner.

**DISCUSSION**

Cultures of brain specimens from patients with MS, ALS, atypical demyelinating disease and leukemia were examined for the presence of viral agents using special techniques for viral detection. In spite of repeated efforts to activate latent viruses by chemical treatment with
BUDR and IUdR and by UV irradiation, no virus was detected. In addition, analysis of sucrose gradients of radioactively labeled culture fluid also failed to reveal any incomplete or defective viruses. With one exception, cocultivation, a sensitive assay for the detection of measles-like agent in subacute sclerosing panencephalitis (4, 10, 16) and JC-type papovavirus in progressive multifocal leukoencephalopathy (6, 13, 18, 19), also produced negative results in this study. The one exception was MS 1 with Vero culture, where the brain cells appeared to exert a transient, inhibitory effect on the initial attachment of Vero cells. Subsequent investigation failed to elucidate this inhibitory activity but revealed another property of the MS 1 brain cells. They were able to slightly stimulate DNA synthesis of Vero cells in a mixed culture. The increase in DNA synthesis, as measured by [H]thymidine incorporation, can be attributed to the presence of a stimulatory factor and/or metabolic products of the brain cells. Tissue culture fluid from the MS 1 brain cultures was similarly stimulatory to Vero cells.

When MS 1 was compared with other human cell cultures, including cultures of muscle, brain, and tumor tissue, two other cultures of brain tissue were discovered to have even greater stimulatory property toward Vero cells. ALS 2 and Rub 1 cultures and their extra-cellular fluids stimulated DNA synthesis in Vero cells. In contrast, brain lines from Leu 1, MS 2, ALS 3, ADD 1, all muscle lines, tumor lines, and human fetal diploid lung had no effect on Vero cells. Significantly, this stimulatory activity was found only in the brain cultures. For example, cultures of muscle tissue from ALS 2 were negative, whereas cultures of the brain tissue were positive for this property.

Preliminary data on the stimulatory factor found in some of the brain cultures are reported here in the hope that they would stimulate investigations from researchers with available brain cultures. Further work is required to elucidate the relationship of growth conditions to the production of the stimulatory factor, and the significance of the presence of the factor in some of the brain cultures. Growth regulation of cells in vitro is ill understood at present. Numerous humoral growth factors have been described in the literature, but only a few have been isolated. Somatomedin C, found in human plasma, is able to stimulate DNA synthesis and mitoses of glial cells in culture (17). Bovine fibroblast growth factor, found in highest concentration in the brain, stimulates DNA synthesis in 3T3 and glial cells (8). Neither the function nor the mode of action of these factors is known.

The negative results in the effort to find evidence of viral agents among brain cultures parallel those of Barbosa and Hamilton (5) in their search for viral agents in MS brain tissues. The results in viral detection are by no means conclusive but should serve to encourage new approaches in the field.

ACKNOWLEDGMENTS

The original specimens from MS 1 and ADD 1 were supplied to Natalie E. Cremer by John Adams. Similarly, tissues from ALS 1, ALS 2, and ALS 3 were from Forbes Norris. Tissues from Rub 1 were from J. R. Baringer. I thank Dr. Cremer for her helpful suggestions and for supplying the frozen cultures established from the brain and muscle tissues.

This investigation was supported by a postdoctoral fellowship from the National Multiple Sclerosis Society and in part by Public Health Service grant AI-01475 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED


