Interferon Production in Cell Lines Derived from Patients with Infectious Mononucleosis (33012)

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Although infectious mononucleosis is presumed to be a viral disease, the etiologic agent has not been isolated. The acute illness is characterized by the appearance of "atypical" lymphocytes in the peripheral blood. Recently we have shown that peripheral leukocytes, obtained from patients with heterophile positive, acute infectious mononucleosis, become established in continuous suspension culture (1). These cell lines are composed of a heterogenous population of immature lymphoid cells which synthesize immunoglobulins and display phagocytic activity. Additionally, cell-free cultures were found to contain an antiviral inhibitor. The present study was undertaken to examine more extensively the nature of the viral inhibitor present in cultured cell lines.

Materials and Methods. Cell lines. Established cell lines which had been in cultivation for at least 2 months were used in this study. Cell suspensions throughout the cultivation period were maintained in RPMI 1640 medium (2) supplemented with 20% fetal calf serum. One hundred ml of 5-day cultures containing approximately 1×10^6 cell/ml were processed by one cycle of freezing and thawing and centrifugation at 2000 rpm for 20 min to remove cellular debris. The supernatant fluid was dispensed in vials and stored at $-70°C$. Prior to use in experiments, stock preparations were tested in rabbits and safety tested to rule out presence of microbial agents and endotoxin (3). Passage fluids from cultures which subsequently proliferated in long-term culture as well as passage fluids from unsuccessful cultures from patients with infectious mononucleosis and normal donors were handled in a similar manner.

Inhibitor assay. Vesicular stomatitis virus (VSV) was assayed in WI-38 cells by the plaque method. In brief, 0.5 ml of serial 0.5 $\log_{10}$ dilutions of test material made in maintenance medium (MM) consisting of 98 parts Eagle's basal medium and 2 parts fetal calf serum was added to duplicate confluent monolayer cultures prepared in 60-mm plastic petri dishes. After 18 hours of incubation at 37°C in 4% CO_2 in humidified air, the cultures were drained and rinsed 3 times with Eagle's medium and then inoculated with 0.5 ml of a dilution containing 30–50 pfu of virus. Following 60 min incubation at 37°C, the fluid was decanted and 5 ml of overlay medium consisting of equal parts of Eagle's tryptose phosphate broth containing 10% fetal calf serum and 1.68% Noble's agar was added to the cultures. After an additional incubation of 24 hours, 2.5 ml of overlay medium containing equal parts of MM and 1.8% Noble's agar with 1% neutral red stain was added and the number of plaques recorded approximately 24 hours later. A reduction of 50% in plaque virus numbers was considered as one unit of interfering activity.

Inhibitor characterization tests. Inhibitor activity was determined in chick embryo fibroblasts by the assay procedure described above. Activity against heterologous virus was measured in WI-38 tube cell cultures. A dilution containing 100 $TCID_{50}$ of Sindbis virus was inoculated into untreated cultures or cultures pretreated for 18 hours with 1.0 ml of undiluted inhibitor preparation. A reduction of 75% or greater in viral cytopathic effect was considered evidence of antiviral activity. Ultracentrifugation of stock preparations was done in a Spinco model L at 105,000 *g* for 90 min. Following centrifugation, the uppermost 4 ml of fluid was removed and saved, the remaining fluid discarded and the pellet resuspended to the original volume with Eagle's medium. Supernatant fluid, pellet, and starting material were tested for activity by the inhibitor

TABLE I. Characteristics of Antiviral Inhibitor in Four Continuous Cell Lines from Two Patients with Infectious Mononucleosis.

Criterion for characterization of inhibitor	Antiviral activity
Assay in homologous cells	Titers ranged from 36 to 72 units/ml
Assay in heterologous cells	No activity
Activity against two unrelated viruses	90% suppression of viral CPE
Ultracentrifugation	Not sedimentable
Treatment with trypsin	Destroyed
Activity in cells pretreated with actinomycin D	70–90% reduction

assay procedure. Stock preparations used for trypsin treatment were adjusted to pH 7.8 and contained 0.2 mg/ml of enzyme. Following incubation at 37°C for 30 min the reaction was stopped by an appropriate concentration of soybean inhibitor. Treated and untreated preparations were tested for antiviral activity by the inhibitor assay procedure. Two ml of inhibitor preparation or MM containing 2 μg/ml of actinomycin D as well as untreated materials were added to duplicate tube cultures of WI-38 cells and placed at 37°C for 4 hours. Following incubation, cell cultures were rinsed 3 times and then inoculated with 10^7 $TCID_{50}$ of VSV. After 60 min of virus adsorption, cultures were rinsed 3 times and fed with 1.5 ml of MM. Eighteen hours later cultures were frozen and thawed, centrifuged to remove cellular debris, and pooled. Samples from control and treated cultures were assayed for virus yield by titration in WI-38 tube cultures.

Results. Inhibitor activity against 30–50 pfu of VSV was demonstrable in undiluted suspensions from each of 8 cell lines derived from 5 patients with infectious mononucleosis. Stock preparations of 4 cell lines from 2 patients were characterized further, and the results of the experiments are summarized in Table I. Each preparation was found to share many properties in common with all interferons, viz., species specificity, antiviral activity is nonspecific for viruses, nonsedimentability, sensitivity to trypsin and loss of activity in cells treated with actinomycin D.

To determine the time of appearance of inhibitor activity in cell lines, harvest fluids were collected weekly from 4 cell lines which subsequently became established in long-term culture. Viral inhibiting activity was detectable in one preparation by 5 weeks of culture and in the other three by 4 weeks. In each case, it appeared that inhibitor activity was demonstrable about the time cellular proliferation became evident with increase in cell numbers, clumping of cells in suspension and acid production. In similar tests with media employed for feeding purposes and harvest fluids from unsuccessful leukocyte cultures from patients with infectious mononucleosis and normal donors, no antiviral activity was detectable in the assay test.

Extensive safety tests for microbial agents and rabbit inoculations for pyrogenic activity showed each preparation to be free of viruses, fungi, bacteria, mycoplasma, and endotoxin.

Discussion and Summary. In a previous report, it has been shown that biopsy material from patients with Burkitt's lymphoma gave rise to continuous cell lines *in vitro* (4,5). The lines appear to be lymphoid in character, produce interferon, contain a herpes-like virus (6,7) and synthesize immunoglobulins (8). In studying the peripheral leukocytes from patients with heterophile positive acute infectious mononucleosis, we have successfully isolated continuous cell lines *in vitro*. These lines possess the biological features of continous Burkitt cell lines (1). In these studies an inhibitor present in the established cell suspensions was identified as interferon on the basis of criteria used for the classification of interferons. Recently it has been reported that lymphoblastoid cells derived from fibroblastic elements of bone marrow of patients with infectious

mononucleosis produced interferon (9). Although the inducer of interferon synthesis has not been identified, preliminary electron microscopy data in our laboratory indicates the presence of unusual 22 mμ particles in all of the cell lines studied for antiviral activity (10).

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Penetration of Red Cell Membranes by Some Membrane-Associated Particles* (33013)

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A novel 100 Å particulate component associated with red cell membranes has recently been demonstrated with freeze-cleave techniques (1). Morphologically similar particles are apparently quite ubiquitous and have been reported on the surfaces of bacterial, fungal, plant, and a variety of animal membranes (2–4). The number and distribution of particles varies for different membranes and some membranes, such as myelin, are free of these particles (5,6). Thus far, speculation on their functional significance has centered on the possibility that these particles may represent multienzyme complexes (2,3) although alternative interpretations have been offered (1,2,6,7).

In preliminary studies on freeze-cleaved and replicated red cells, membrane-associated particles were observed on both outer and inner surfaces of the red cell membrane(1). However, the quality of these earlier replicas precluded examining the membranes in cross section. Replicas made with an improved freeze-cleave and replication apparatus used in this study now allow for the examination of the relationship of these particles to membrane ultrastructure as seen in cross section. These improved replicas show that some, but not all, cell membrane-associated particles penetrate through the entire thickness of the red cell membrane forming continuous structural units between the external milieu and the cell's cytoplasm.

Methods. Improved freeze-cleave apparatus. In these epxeriments, a modified Bullivant–Ames freeze-cleave and replication apparatus(7) was used to produce replicas of frozen fracture faces of intact red cell membranes and red cell ghosts. The original Bullivant–Ames specimen block ("type I apparatus") consists of a cylindrical brass block with the specimen well located on its upper face(7). A heavy brass lid covers the specimen well when the brass block is carried to an evaporator for replication with carbon and carbon–platinum following fracturing of the

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