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VIRAL RESISTANCE AND INTERFERON SYNTHESIS IN CELL LINES DERIVED FROM BURKITT'S LYMPHOMA AND LEUKEMIC TISSUES

BY

Barbara Ann Zsjac

A DISSERTATION

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ABBREVIATIONS

FCS	Fetal calf serum
HBSS	Hanks balanced salt solution
HBSS-2	HBSS supplemented with 2% FCS
HBME	Basal medium Eagle in HBSS
HBME-2, 5, 10	HBME supplemented with 2%, 5%, and 10% FCS respectively.
MEM	Minimum essential medium
HMEM	MEM in HBSS
HBME-HMEM	Combination medium, 47.5% of each
1629-2, 10	Medium 1629 supplemented with 2% and 10% FCS respectively, 1629-2 is referred to as maintenance medium
Medium 199	Consists of 30% Parker solution 199, 60% Scherer maintenance medium, and 10% inactivated horse serum
PBS	Phosphate-buffered saline
HEK	Human embryonic kidney cells
GMK	Green monkey kidney cells
L(MCN)	L cells, mouse fibroblasts
RK	Rabbit kidney cells
HSV	Herpes simplex virus
VSV	Vesicular stomatitis virus
NDV	Newcastle disease virus
NDV_{uv}	NDV inactivated by ultraviolet irradiation
CPE	Cytopathogenic effect
EID_{50}	50% egg infectious dose
FA	Fluorescent antibody
FITC	Fluorescein isothiocyanate
HAU	Hemagglutinating unit
I.m.	Input multiplicity

LD_{50}	50% lethal dose
PFU	Plaque forming unit
TCD_{50}	50% tissue culture dose
VP	Virus particles
$EB\text{-}1_{mumps}$	EB-1 Burkitt lymphoma cells infected with mumps virus

EXPLANATION OF TABLE 1

(1) FCS, fetal calf serum; HBME, basal medium Eagle in Hanks balanced salt solution.

(2) Herpes-type virus particles detected by electron microscopy.

(3) Fluorescent antibody studies, Henle and Henle, 1966a, Henle et al., unpublished.

(4) Epstein et al. (1964a).

(5) Epstein et al. (1966b).

(6) Personal communication.

(7) No detectable herpes-type virus particles were found in SK-L1, SK-L2, LK-1D, LK-57, LK-60, and 6410 cell lines (Hummeler, personal communication).

(8) Very rare + cells, specificity questionable.

(9) Iwakata and Grace (1964) reported presence of herpes-type virus particles. Electron microscopic studies performed at a later date indicated no detectable virus (Grace, personal communication).

(10) Not done.

EXPLANATION OF TABLE VIII

(1) Herpes-type virus particles detected by electron microscopy.

(2) Fluorescent antibody staining.

(3) 24 units or less/ml.

(4) Not done.

(5) 100 units-300 units/ml.

(6) 500 units or greater/ml.

(7) No detectable herpes-type virus particles were found in SK-L1, SK-L2, LK-57, LK-60, and 6410 cell lines (Hummeler, personal communication).

(8) Very rare $^{+}$ cells, specificity questionable.

(9) Ikawata and Grace (1964) reported presence of herpes-type virus particles. Electron microscopic studies performed at a later date indicated no detectable virus (Grace, personal communication).

INTRODUCTION

Burkitt's or African lymphoma aroused considerable interest because epidemiologic observations provided circumstantial evidence that it might possibly be caused by a virus (Burkitt, 1962). Patients often develop multiple tumors at several sites which could be due to simultaneous seeding of an infectious agent; clusters of cases have been observed indicative of a common source of infection (Burkitt and Wright, 1966; Wright, 1967); and the geographic distribution of the cases concurs with the spread of various arthropods suggesting that a vector might play a role in the dissemination of the disease (Burkitt, 1963), that is, Burkitt's lymphoma is seen almost exclusively in African regions with temperatures not less than 60 F, annual rainfall exceeding 20 inches, and altitudes below 5000 feet. Cases of Burkitt's lymphoma are apparently not restricted to Africa however, and tumors histologically indistinguishable from the African lymphoma have been observed in England (Epstein et al., 1966a; Wright, 1966), New Guinea (ten Seldman et al., 1966), South America (Beltran et al., 1966), and the United States (Dorfman, 1965; O'Conor et al., 1965).

The suggestion of an insect vector, transmitting presumably a virus, has sparked extensive efforts to isolate infectious agents from biopsy materials. Indeed, herpes simplex virus (Haddow and Woodall, 1962; Simons, 1962; Simons and Ross, 1963; 1965; Woodall et al., 1965) and reovirus and reovirus-like agents (Bell et al., 1964; 1965; 1966) have been isolated repeatedly from tumor specimens as well as mycoplasma (Dalldorf et al., 1966). No irrefutable evidence has been presented to link anyone of these ubiquitous agents to the etiology of Burkitt's tumor. However, Stanley (1966) has proposed a hypothesis that Burkitt's lymphoma might be caused by reovirus type 3. This virus has been isolated from mosquitoes and its insect transmission amongst Australian vertebrates is almost certain (Parker et al., 1965). In addition Stanley et al. (1966) reported that neonatal infection of mice with reovirus 3 produced a late chronic runting syndrome. When suspensions of splenic cells from "runts" were inoculated into infant mice, malignant lymphomas developed in a certain proportion of these animals which were histologically indistinguishable from Burkitt's tumor. On electron microscopic examination of such murine lymphomas, Papadimitriou (1966) observed the presence of virus particles which resembled herpes rather than reovirus.

A number of cell lines have been established from biopsy specimens and maintained in continuous culture (Epstein and Achong, 1965; Epstein and Barr, 1964; 1965; Epstein et al., 1964b; 1965b; 1966a, b; Pulvertaft, 1964; 1965; Stewart et al., 1965; Rabson et al., 1966; Osunkoya, in press). All these cultures are composed of lymphoblasts but they differ among each other by the degree of immaturity of the cells. Chromosomal analyses have shown that the lymphoblasts have retained their diploid character even after cultivation for as long as

3-4 years (Kohn et al., 1967; Miles and O'Neill, 1967). These cells do not attach to the surface of culture vessels but grow freely in the fluid phase. When seeded onto monolayer cultures of human or certain animal cells the lymphoblasts may attach to the cell sheets, and then grow in depth in the form of macroscopically visible hillocks (Henle and Henle, 1965a).

It was of particular interest that many of these cultures, as first observed by Epstein, harbored a herpes-type virus (Epstein et al., 1964a; 1965a,b; 1966a). Subsequently similar virus particles were noted to be present in a lymphoblast line from a lymphoma of an American patient (O'Conor and Rabson, 1965), in cell cultures derived from leukemic tissues or from lymphnodes with metastatic neoplasias (Iwakata and Grace, 1965; Moore et al., 1966; Zeve et al., 1966; Minawada et al., to be published), and in continuous lines of peripheral leucocytes of healthy donors (Moore et al., 1967; Gerber, personal communication). Herpes-type virus particles have been seen also in thin sections of a few Burkitt tumor biopsies (Griffin et al., 1966).

The size and configuration of the virus particles found in sectioned cells suggested that they belong in the herpes group of viruses. Additional studies employing the negative contrast technique (Hummeler et al., 1966; Toplin and Shidlovsky, 1966) revealed the virus particles to be morphologically indistinguishable from members of the herpes group; i.e., they represented icosahedra, either free or enclosed in an envelope, with a capsid composed of 162 capsomers. The vast majority of these particles were defective, however, in that they lacked internal structure, presumably all or part of the viral nucleic acid.

The high incidence of defective particles offered at least a partial explanation for past failures to transmit the agent to various types of primary or continuous cell cultures of human or animal origin, to chick embryos, or to newborn and weanling animals (Epstein et al., 1965c). Judging by the numerous examples described in the literature, many viral infections in vivo or in vitro, including oncogenic viruses, remain subclinical or latent and may persist for long periods of time. In some established persistent viral infections, especially those of long duration, transmission of the carried agent to other host systems may be unsuccessful or achieved only with great difficulty because largely defective viruses or even only viral antigens are produced by the cells. Demonstration of the presence of virus under such conditions may depend then upon electronmicroscopic examination of sections of tissues or cultured cells, application of virus-specific immunofluorescence, tests for interference with normally cytopathic viruses, or other indirect evidence of viral activities.

Since only a small proportion of cells in various Burkitt or other cell lines is actually producing herpes-type virus particles, monitoring of the extent of the infection by electron microscopy has been cumbersome. The discovery that infected cells can be detected readily by immunofluorescence techniques has overcome this handicap (Henle and Henle, 1966a). In indirect immunofluorescence tests positive results were obtained regularly with sera from Burkitt patients and also with many sera from African or American control individuals. The antibody was found in American adult sera with sufficient frequency and in sufficient titer to render fluorescein-conjugated human gamma globulins suitable for direct staining of virus-producing Burkitt cells. On the basis of appropriate virus-specific immunofluorescence tests, the virus could not be identified as herpes simplex, varicella, cytomegalo, or any one of 8 different animal herpes viruses (Henle and Henle, 1966b).

The conclusion that the immunofluorescence techniques, as employed, detect those Burkitt cells which harbor the herpes-type virus is supported by the following evidence: (a) electron microscopic estimates of the percentage of virus-containing cells in the various lines closely matched the percentage of cells showing immunofluorescence (Henle and Henle, 1966a, b; Hinuma et al., personal communication); (b) viral inhibitors or cultural manipulations which reduced or increased the percentage of fluorescent cells equally decreased or raised the number of virus-containing cells as determined by electron microscopy (Henle and Henle, 1966b); (c) sera which were strongly positive in indirect immunofluorescence tests, but not negative sera, were found to contain antibodies to the viral capsid as shown electron microscopically by antibody coating and agglutination of virus particles extracted and concentrated from cultured cells (Henle et al., 1966; Mayyasi et al., personal communication); (d) 24-48 hours following a pulse exposure of lymphoma cells to tritiated thymidine the majority of fluorescent cells (up to 80%) were shown by autoradiography to contain cytoplasmic label, whereas non-fluorescent cells were less frequently labeled, and then in the nucleus (zur Hausen et al., 1967); and (e) picked individual fluorescent cells after embedding and thin sectioning when examined electronmicroscopically were found to contain numerous virus particles, whereas none were seen in non-fluorescent cells prepared in the same manner (zur Hausen et al., 1967). The evidence clearly indicated that the immunofluorescence described was largely due to viral antigens in the cells and not due to cellular membrane antigens detected by other workers (Klein et al., 1966; Benyesh-Melnick et al., personal communication) using indirect immunofluorescence tests with live cells.

On the basis of serological surveys by the indirect immunofluorescence technique, it is evident that the herpes-type virus found in cultured Burkitt cells, or a close relative of it, is widely disseminated not only in Africa but also in the United States and probably elsewhere. This does not necessarily exclude the possibility that the virus might be etiologically related to Burkitt's tumor since (a) Burkitt's lymphoma has been observed in various parts of the world; and (b) known oncogenic viruses may induce tumors only rarely under natural conditions.

Certain non-cytopathic viral infections of cell cultures can be detected by the resistance they may engender to other, usually destructive viruses. In many instances the resistance may be due to classic interference and interferon synthesis, but in others to blockade of cell receptors, metabolic deficiencies, or as yet unknown factors (cf. Walker, 1964). Thus, tests for viral interference or interferon have become routine procedures in the search for suspected latent viral infections of cell cultures. The first 3 available Burkitt tumor cell lines (EB-1, EB-2, Sl_1), all of which were known to harbor herpes-type virus, behaved in every respect like viral carrier populations in which interference and interferon synthesis limits the spread of the infection and thus permits both virus and cells to persist simultaneously in culture for long periods of time (Henle and Henle, 1965 a,b). The Burkitt cell lines were markedly resistant to vesicular stomatitis virus (VSV), transmitted the resistance to other human cells on mixed cultivation, and released an inhibitor in low concentration into the culture media which appeared to be an interferon. In contrast, the RPMI-6410 line, which was isolated by Iwakata and Grace (1964) from a leukemic patient and found free of virus at the time of study, succumbed rapidly to infection by VSV and failed to produce an inhibitor. Thus, all the evidence suggested that the herpes-type viral carrier state in Burkitt tumor cell lines was controlled by interference and interferon synthesis.

The work to be reported in this thesis is divided into 3 sections, all of which are concerned with aspects of the cellular resistance referred to above. In Section I results are recorded of an extension of the tests for a viral carrier state to additional Burkitt cell lines as well as continuous cultures of hematopoietic cells derived from leukemic patients or healthy donors. Some of these were known to harbor herpes-type virus and others not. The studies revealed that the resistance of some of the cultures to extraneous viruses as well as the production of the interferon-like inhibitor are apparently unrelated to a herpes-type viral carrier state. Since some cell lines synthesize the inhibitor without evident stimulation, it is being termed autogenous interferon.

In Section II autogenous and virus-induced interferons, derived in part from the same cell lines, are compared with respect to optimal conditions for production and their physical, chemical, and biological properties. While generally no significant differences were observed, it became clear that the presence of autogenous interferon enhances the time of appearance and rate of virus-induced interferon synthesis. The susceptibility of some autogenous interferon-producing cultures to extraneous viruses was found to be explainable on the basis that too small quantities of autogenous interferon were produced. Such cultures could readily be protected by addition of homologous concentrated autogenous interferon.

Section III presents attempts to lower the resistance of Burkitt cell lines in order to determine whether infection by the indigenous herpes-type virus could be increased by such means. Since members of the paramyxovirus group are known to reduce resistance of various cell populations to a number of unrelated viruses (Kumagi, 1958; Frothingham, 1963; Hermodsson, 1963; Kato et al., 1965; Valle and Cantell, 1965; Maeno et al., 1966), Burkitt cell cultures were superinfected with mumps virus. The superinfection did indeed increase their susceptibility to vesicular stomatitis and herpes simplex viruses and prevented the protective action of added extraneous human interferon. It did not influence autogenous interferon synthesis but appeared to reduce virus-induced interferon production. Yet, the extent of the herpes-type viral infection was not affected by the change in the resistance of the cultures.

MATERIALS AND METHODS

I. Cell Cultures.

A. Lymphoblast lines derived from Burkitt's tumor and other hematopoietic cell lines derived from patients with leukemia, non-malignant diseases, or healthy donors:

1. Cell lines, media, and presence or absence of herpes-type particles: Table I lists all the cell lines used in this study, their origins and authors, the media used for growth of the cells in this laboratory, and the presence or absence of cells producing herpes-type virus particles as detected by electron microscopy or immunofluorescence. All the media used were supplemented with 100 units penicillin, 100 µg streptomycin, and 2mM glutamine per ml. Fetal calf serum (FCS) was inactivated at 56 C for 30 minutes. For certain experimental procedures the cells were transferred to a maintenance medium in which the FCS concentration was reduced to 2% (1629-2). Medium RPMI 1629 was purchased from Baltimore Biological Laboratories, Baltimore, Maryland, and other media and FCS from Microbiological Associates, Inc., Bethesda, Maryland. For convenience throughout the text lymphoblast lines derived from Burkitt's tumor and lines derived from patients with leukemia will be referred to as Burkitt lymphoma and leukemia cell lines, respectively.

2. Preparation of stock cultures: The concentration of cells was determined microscopically by aid of a hemacytometer. Trypan blue was routinely added to give a final concentration of 0.1%, to permit

TABLE I

Cell lines, culture media, and presence or absence of herpes-type virus particles*

Cell line	Source	Reference	Medium(1)	VP(2)	FA(3)
EB-1	Burkitt's lymphoma	Epstein and Barr (1964)	1629(90%), FCS(10%)	+4	+
EB-2	Burkitt's lymphoma	Epstein et al. (1964b)	HBME(90%), FCS(10%)	+	+
EB-3	Burkitt's lymphoma	Epstein et al. (1965b)	1629(90%), FCS(10%)	+	+
EB-4	Burkitt's lymphoma	Epstein et al. (1966a)	1629(90%), FCS(10%)	-	-
Raji	Burkitt's lymphoma	Pulvertaft (1964)	1629(90%), FCS(10%)	-(5)	-
Ji joye	Burkitt's lymphoma	Osunkoya and Pulvertaft(6)	1629(90%), FCS(10%)	+	+
Kudi	Burkitt's lymphoma	Osunkoya and Pulvertaft(6)	1629(90%), FCS(10%)	+	+
Ogun	Burkitt's lymphoma	Osunkoya and Pulvertaft(6)	1629(90%), FCS(10%)	±	±
SL1	Burkitt's lymphoma	Stewart et al. (1965)	HBME(90%), FCS(10%)	+	+
SK-L1	Monocytic leukemia	Clarkson et al. (to be published)	1629(90%), FCS(10%)	-(7)	(±)(8)
SK-L2	Lymphoblastic leukemia	Clarkson et al. (to be published)	1629(90%), FCS(10%)	-	(±)
LK-1D	Lymphoblastic leukemia	Armstrong (1966)	1629(80%), FCS(20%)	-	(±)
LK-57	Myeloblastic leukemia	Armstrong (1966)	1629(80%), FCS(20%)	-	-
LK-60	Lymphoblastic leukemia	Armstrong (1966)	1629(80%), FCS(20%)	-	-
6410	Myeloblastic leukemia	Ikawata and Grace (1964)	1629(90%), FCS(10%)	+(9)	-
L265	Myeloblastic leukemia	Moore et al. (1966)	1629(80%), FCS(20%)	n.d.(10)	-
7666	Healthy donor	Moore et al. (1967)	1629(80%), FCS(20%)	+	±
7466	Acute Rheumatic Arthritis	Moore et al. (1967)	1629(80%), FCS(20%)	+	±

*Refer to page xxvi for explanation of table.

determination of the number of cells which were viable. In the presence of trypan blue, cells which had lost their selective permeability properties and adsorbed dye, were considered dead.

The suspension cultures were maintained by dilution in fresh medium every 5-7 days. The number of viable cells per ml was determined and the density of the culture was readjusted to 1×10^5 cells per ml by removing aliquots of cell suspension, transferring them to fresh flasks, and diluting with fresh growth medium. Cells were grown in Erylenmeyer flasks at 37 C with a medium to air ratio of 1 to 4.

B. Peripheral blood cells: Fresh human leucocyte preparations were kindly supplied by Dr. M. Miller, The Children's Hospital, Philadelphia. These were suspended in medium 1629-10 and utilized on the day they were obtained.

C. Primary and continuous cell lines utilized for assays:

1. Primary cultures: Human embryonic kidney (HEK) and green monkey kidney (GMK) tube cultures were obtained from Flow Laboratories, Rockville, Maryland, or Microbiological Associates, Inc., Bethesda, Maryland. Human diploid cell cultures (strain WI-38) were obtained from the Wistar Institute, Philadelphia. Primary rabbit kidney cell cultures were kindly supplied by Dr. T. Tokumaru, The Children's Hospital, Philadelphia.

2. Continuous cell lines: L(MCN) cell cultures (McCulloch, E.A., 1955; Rothfels et al., 1959) were grown and maintained as described by Rodriguez and Henle (1964). Fat head minnow cells were kindly supplied by Dr. R. Malsberger, Lehigh University, Allentown, Pennsylvania. Turtle heart cell cultures were obtained through the courtesy of Dr. D. T. Karzon, Children's Hospital, Buffalo, New York.

3. Maintenance of the cells: All the above cell cultures, with the exception of L(MCN) cells, were grown on Eagle basal medium in Hanks balanced salt solution, containing 10% inactivated FCS, 100 units penicillin, 100 µg streptomycin, and 2mM glutamine per ml (HBME-10). The L(MCN) cells were grown on a medium consisting of 30% Parker solution 199, 60% Scherer maintenance solution, 10% inactivated horse serum, and antibiotics (Medium 199).

II. Viruses.

A. Vesicular stomatitis virus: The Indiana strain of VSV which had been obtained from the American Type Culture Collection and passed twice in L(MCN) cells and seven times in chick embryo fibroblasts was used. Virus pools were prepared in chick embryo fibroblast cultures incubated at 37 C. When cytopathic effects became evident in more than half of the cells, the cultures were frozen and thawed 1x, and the suspension was centrifuged at 4 C at 1500 rpm for 15 minutes. The supernatant was shell frozen in ampules and stored at -70 C in a dry ice cabinet. The infectivity titers of the VSV stocks were 1.0×10^9 to 1.6×10^9 plaque forming units (PFU) per ml when assayed on L(MCN) cell monolayers.

B. Mumps virus: The Ricki strain of mumps in its 6th amniotic passage was used. It had been isolated in this laboratory. Virus pools were prepared by amniotic inoculation of 8 day-old embryonated chicken eggs. The eggs were incubated at 37 C for 6 days and the amniotic fluids were harvested. Fluids exhibiting hemagglutination on spot check were pooled and centrifuged at 1500 rpm for 10 minutes. The supernatant was shell frozen in ampules and stored at -70 C. The infectivity titers of the mumps virus stocks ranged from $10^{6.2}$ to $10^{7.7}$ TCD_{50} per ml when assayed in HEK monolayers. The hemagglutinating titers measured with chicken erythrocytes ranged from 3.2×10^{2} to 1.3×10^{3} HAU per ml.

C. Newcastle disease virus: The Victoria strain of NDV propagated for an undetermined number of allantoic passages in embryonated eggs was kindly supplied by Dr. K. Paucker. The infectivity titers in embryonated eggs ranged from 1.0×10^{9} to 1.6×10^{10} EID_{50} per ml. This virus was employed active or after inactivation by ultraviolet light.

Ultraviolet irradiation of NDV was carried out as described by Paucker and Boxaca (1966). NDV-containing allantoic fluids were dialyzed overnight at 4 C against 20 volumes of calcium-and magnesium-free phosphate-buffered saline (PBS) (Dulbecco, 1954) at pH 7.0. The dialyzed materials were exposed for 20 seconds in 20 ml amounts in Petri dishes (100 x 15 mm) to a 15 watt germicidal lamp at a distance of 7 inches. The plates were tilted up and down mechanically at a rate of 90 times per minute with excursions of 1 inch. Virus treated in this manner is referred to as NDV_{uv}.

D. Other viruses: Kindly supplied by Dr. F. Lief, Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Philadelphia were: (1) the 213 strain of Sendai virus in its 5th egg passage; (2) the PR8 strain of influenza Type A virus in its 24th egg passage; (3) the A_2/2496 strain of influenza virus in its 17th egg passage; and (4) the Johannesburg strain of influenza Type B virus in its 10th egg passage. All these viruses were propagated in the allantoic cavity of embryonated eggs. The infectivity titer of Sendai virus was 6.3 x 10^8 EID_{50} per ml in embryonated eggs. The hemagglutinating titers of the three influenza strains measured with chicken erythrocytes ranged from 5.1 x 10^3 to 1.0 x 10^4 HAU per ml.

Sindbis virus grown in chick embryo fibroblasts was obtained from Dr. K. Paucker. The infectivity titer was 2.5 x 10^8 PFU per ml in chick fibroblasts cultures.

Vaccinia virus grown in HEK cultures was supplied by Dr. G. Henle. The infectivity titer was 1.6 x 10^7 TCD_{50} per ml in HEK monolayers.

St. Louis and California encephalitis viruses were kindly supplied by Dr. S. Mazzur, New Jersey Department of Public Health, Trenton. Both viruses were propagated by intracerebral inoculation of weanling mice. The infectivity titers were 10^8 and 10^6 LD_{50} per ml, respectively.

III. Infectivity Assays.

A. Assays for infectious VSV:

1. Tissue culture dose: Suspensions of L(MCN) cells were prepared as described (Rodriguez and Henle, 1964). One ml volumes of medium containing 1 x 10^5 cells were transferred to tissue culture tubes, and incubated at 37 C for 48 hours. The tissue culture tubes were refed with 1.0 ml of Medium 199. The pH of the medium was adjusted to 7.6 with a 7.5% solution of sodium bicarbonate. Two to four tubes

were inoculated with 0.2 ml of one of several ten-fold dilutions of the virus in Hanks balanced salt solution (HBSS). The extent of virus-specific cytopathic effect in each tube was recorded one, two, and three days after inoculation. The infectivity titers were calculated as TCD_{50} per ml by the method of Reed and Muench using the third day recordings.

2. Plaque forming units: Five ml volumes of medium containing 3×10^6 cells were transferred to plastic flasks (Falcon Plastic Company, Los Angeles, California), and incubated at 37 C for 48 hours. At this time the monolayers had formed and the medium was discarded. Two to four flasks were inoculated with 0.4 ml of one of several ten-fold dilutions of the virus in Medium 199, and incubated at 37 C for one hour. Thereafter the monolayers were overlayed with 8 ml of medium consisting of 30% Parker solution 199, 50% Scherer maintenance solution, 20% inactivated horse serum, 1.2% Difco agar, neutral red 1:40,000 (final concentration), and penicillin and streptomycin. The pH was adjusted to 7.6 by the addition of sodium bicarbonate. Plaques were counted after 2 days of incubation at 37 C, and virus concentrations were expressed as the number of PFU per ml of original volume.

B. Assay for infectious mumps virus: HEK tissue culture tubes were refed with 1.0 ml of HBME-5, pH 7.4. Two to four tubes were inoculated with 0.2 ml of one of several ten-fold dilutions of the virus in HBME-5 medium and incubated at 37 C. The extent of mumps infection was determined on day 6 by hemadsorption. The infectivity titer was calculated as TCD_{50} per ml by the method of Reed and Muench.

C. Hemagglutination assays:

1. Hemagglutinin titrations: These assays were carried out as described by Henle et al., (1958).

2. Hemadsorption with chicken erythrocytes: The technique was described by Vogel and Shelokov (1957). To each drained tissue culture tube was added 0.5 ml of a chilled 1% suspension of washed chicken erythrocytes in veronal-buffered saline. The tubes were placed at 4 C for 30 minutes and examined microscopically for the extent of hemadsorption. Any tube showing a single area of hemadsorption was considered infected.

IV. Sera.

A. Vesicular stomatitis virus antisera: Virus pools for immunization were prepared by allantoic passage of the seed stock. The embryonated chicken eggs were incubated at 37 C for 24 hours. The allantoic fluids were harvested, pooled, and centrifuged at 1500 rpm for 10 minutes and stored at 4 C. Fresh allantoic fluids were prepared prior to each immunization, using fresh ampules of the stock seed.

Rabbits were inoculated intraperitoneally (i.p.) at weekly intervals with 8 to 10 ml volumes of virus suspension. Ten days after the last of 4 doses the rabbits were bled. Seven days later they were given a booster injection of 10.0 ml of virus i.p. Starting one week after the first booster, the rabbits were bled at weekly intervals and 24 hours later again boosted. This procedure was repeated for seven weeks. Blood was collected by cardiac puncture and the sera were separated, inactivated at 56 C for 30 minutes, and stored at -20 C.

The VSV neutralizing titer was calculated as 50% plaque inhibiting units per ml using the reciprocal of the highest dilution of antiserum inhibiting at least half of the plaque forming units present in the monolayers with no serum. The antibody titers obtained ranged from 1.0×10^4 to 2.0×10^4 50% plaque inhibiting units per ml as assayed on L(MCN) monolayers using 100 PFU of VSV.

B. Mumps virus antisera: Fluorescein isothiocyanate (FITC)-conjugated human gamma globulin with high titers of antibodies to mumps was kindly supplied by Drs. G. and W. Henle. This conjugate also contained antibodies to the herpes-type virus present in cultured Burkitt lymphoma cells (Henle and Henle 1966a).

Equine anti-mumps serum was kindly supplied by Dr. M. Hilleman, Merck Institute for Therapeutic Research, West Point, Penna. The mumps neutralizing titer was determined using the neutralization-hemadsorption technique described by Shelokov et al., 1958. The number of tubes showing inhibition of hemadsorption was recorded and the 50% neutralizing endpoint of the mumps antiserum was calculated by the method of Reed and Muench. This serum contained about 1.0×10^4 50% TCD inhibiting units per ml when assayed in HEK monolayer cultures using 100-200 TCD_{50} of mumps.

C. Newcastle disease virus antiserum: FITC-conjugated rabbit gamma globulin with high titers to NDV (Rodriguez and Henle, 1964) was kindly supplied by Dr. W. Henle.

D. Herpes-type virus antibodies: Antibodies against the herpes-type virus present in cultured Burkitt lymphoma cells were provided by commercially produced pools of human gamma globulin.

V. Interferon: Production, Concentration, and Assay

A. Production:

1. Autogenous interferon: Cell suspensions containing 1×10^7 cells per ml in 1629-2 were incubated on a rotray shaker at 37 C for 24 hours. The media were harvested and, following centrifugation at 1500 rpm for 10 minutes, stored at 4 C. The autogenous interferon samples were routinely assayed without further treatment.

2. Virus-induced interferon: Samples of cell suspensions containing 1×10^8 cells were centrifuged at 1000 rpm for 10 minutes. The pellets were resuspended in 5.0 ml of medium containing a given multiplicity of inactivated virus, or in 1.0 ml of medium containing a given multiplicity of viable virus. The virus was allowed to adsorb for one hour at 37 C. Following adsoprtion viable virus was removed by centrifugation, the cell concentrations were adjusted to 1×10^7 per ml with maintenance medium, and the cultures incubated on a rotary shaker at 37 C for 24 hours. The media were harvested and, following centrifugation, dialyzed under sterile conditions for 24 to 72 hours at 4 C against 100 volumes of HCL-KCL buffer at pH 2.0, and then for another 24 hours against 100 volumes of PBS at pH 7.0. After the last dialysis the interferon samples were stored at 4 C.

B. Concentration: All interferons were concentrated at 4 C by ultrafiltration using 0.25 inch Visking tubing under vacuum (Schonne, 1966). After a 5- to 20-fold concentration, as determined by volume recovery, the materials were dialyzed against 100 volumes of PBS for 24 hours. The dialyzed materials were stored at 4 C.

C. Assays:

1. Autogenous interferon: HEK tube cultures were refed with

1.0 ml of maintenance medium (HMBE-2, pH 7.6). Serial 2-fold dilutions of the interferon sample were made in HBSS-2 and 0.5 ml aliquots were added to duplicate cultures of HEK cells. After 24 hours incubation at 37 C the tubes were challenged with 0.2 ml of a VSV suspension containing 1000 TCD_{50}. The cultures were incubated at 37 C for 40 hours at which time the controls showed total destruction of cells. The endpoint was taken as the highest initial dilution of interferon sample which gave 50% suppression of viral cytopathic effect. One unit of interferon was 1.0 ml of the greatest dilution (2 times the endpoint) of the sample which caused 50% suppression of VSV cytopathology in the assay.

2. Virus-induced: HEK cultures were inoculated with viral interferon as described above for autogenous interferon except that the interferon was removed and the cells refed with 1.5 ml of fresh medium prior to challenge with VSV.

VI. Assay for Virus Attachment.

A. Attachment of VSV to Burkitt lymphoma cells: Cell suspensions containing 5×10^6, 3×10^7, 1×10^8 cells, were centrifuged at 1000 rpm for 10 minutes and the sedimented cells resuspended in 1.0 ml of VSV (2.0×10^7 PFU) diluted in phosphate buffer (pH 7.8) containing 2% FCS. The mixture was incubated in a water bath at 37 C for 1 hour. At 15, 30 and 60 minute intervals a 0.1 ml aliquot of cell-virus suspension was diluted 1:100 into 10.0 ml of Medium 199, to stop viral attachment, and the diluted cell suspension centrifuged at 1800 rpm for 10 minutes to remove the cells. The upper 5.0 ml of the supernatant fluid containing unattached virus was shell frozen in ampules and stored at -70 C for virus assay. For reference of initial virus content, a virus control tube containing only the virus inoculum was included.

The amount of unattached virus was determined by plaque assay on L(MCN) monolayers. Each of three prepared cultures were inoculated with 0.4 ml of serial 10-fold dilutions of sample. Those cultures yielding 25-50 PFU were used for calculation of the titer. Virus concentrations were recorded as the number of PFU per ml of original suspension.

VII. Immunofluorescent Technique.

A. Preparation of fluorescein conjugate: The gamma globulin fractions of the various anti-sera were separated by ammonium sulphate precipitation and coupled to fluorescein isothiocyanate by conventional methods (Marshall et al., 1958; Riggs et al., 1958). The labeled gamma globulin was adsorbed twice with mouse liver powder (Coonset al., 1955) before use.

B. Preparation of coverslips for fluorescent antibody staining:

1. Suspension cultures: In order to determine the number of cells staining in a given population, 1×10^6 cells were centrifuged, the supernatant decanted, and the moist pellet spread with a Pasteur pipette over 3 coverslips (6 x 30 mm) and dried at 37 C.

2. Coverslip tube cultures: In certain assay procedures, cells grown on coverslips (6 x 30 mm) were used for fluorescent antibody studies. Trypsinized cells were diluted in HBME-10 and tissue culture tubes containing floating coverslips were seeded with 1.0 ml of the cell suspension. The cultures were incubated at 37 C. Infected coverslips removed from tissue culture tubes were washed in PBS and thoroughly dried at 37 C.

3. Fixation and staining of prepared coverslips: All coverslips were fixed in acetone at room temperature and stored at -20 C. Staining with fluorescent conjugate proceeded at 37 C for 1 hour and after thorough washing in PBS and two rinses in distilled water the coverslips were mounted on slides with a semipermanent mounting medium (Rodriguez and Deinhardt, 1960). Mounted coverslip cultures were examined for the presence of specific intracellular fluorescence under ultraviolet illumination (Osram HBO 200-w lamp) with a Zeiss microscope. The percentage of cells showing virus-specific immunofluorescence was determined by counting 300 to 400 cells.

VIII. Other Techniques.

A. Shaking of cultures: Continuous agitation of cultures was provided by a Thomas rotator (Arthur H. Thomas, Co., Philadelphia) at a setting of 125 rpm. This rotator provided a gentle swirling motion which did not permit the cells to settle to the bottom of the container.

B. Sonication of Burkitt lymphoma cells: One ml aliquots of cell suspension were transferred to 11 x 100 mm sterile test tubes stoppered and sonicated in an ultra sonic unit (DiSontegrater 40, Ultrasonic Industries, Inc., Plainfield, Long Island, New York) at 80 kc per second for 3 minutes.

RESULTS

I. Evaluation of Various Biological Assays for Detection of the Herpes-Type Viral Carrier State.

The ability of cell cultures to sustain persistent viral infections in the absence of readily detectable cytopathic effects is by no means unusual and has been studied by many investigators. This subject has been recently reviewed by Walker (1964). A number of distinct mechanisms may be responsible for different viral carrier states. Prominent among these are induction of interference and synthesis of interferon which protect part of the cell population and thus account for the maintenance of the carrier state. An example of this type of persistent viral infection is provided by L(MCN) cell cultures infected with Newcastle disease virus (Henle et al., 1958; Bergs et al., 1958; Deinhardt et al., 1958; Henle et al., 1959; Henle, 1963; Rodriguez and Henle, 1964). This L_{NDV} model has provided certain criteria applicable to analysis of other viral carrier states based upon a similar mechanism. These criteria are as follows: (a) L_{NDV} cultures are markedly, though not absolutely resistant to vesicular stomatitis virus (VSV); (b) the resistance to VSV is readily transferred by carrier cells to recipient stock L cell populations on co-cultivation; (c) the carrier cultures produce low levels of interferon; (d) they produce additional, though low titers of interferon on appropriate stimulation by extraneous virus; and (e) addition of exogenous interferon enhances the resistance of carrier cultures to VSV. Applying the above criteria Henle and Henle (1965a, b) demonstrated that 3 lines of Burkitt lymphoma cells (EB-1, EB-2, and SL_1) shown to contain a herpes-type virus (Epstein et al., 1964a, b; Stewart et al., 1965), behaved in every aspect like a L_{NDV} carrier culture. In order to determine whether these observations were truely referable to the herpes-type viral carrier state these studies were extended to additional Burkitt cell lines, some of which were free of this agent, as well as to cell lines derived from leukemic patients.

A. Resistance to vesicular stomatitis virus: Burkitt lymphoma cell lines were challenged with VSV, in order to determine whether those harboring herpes-type virus particles would always be resistant, and those free of the agent would be susceptible to the challenge virus. Leukemia cell lines were included because some of these have been reported to harbor herpes-type virus. Vesicular stomatitis virus was used as the challenge agent because of its broad host cell spectrum. Of numerous primary and continuous cultures tested earlier, all were rapidly and completely destroyed by VSV regardless of the species of origin and type of cell.

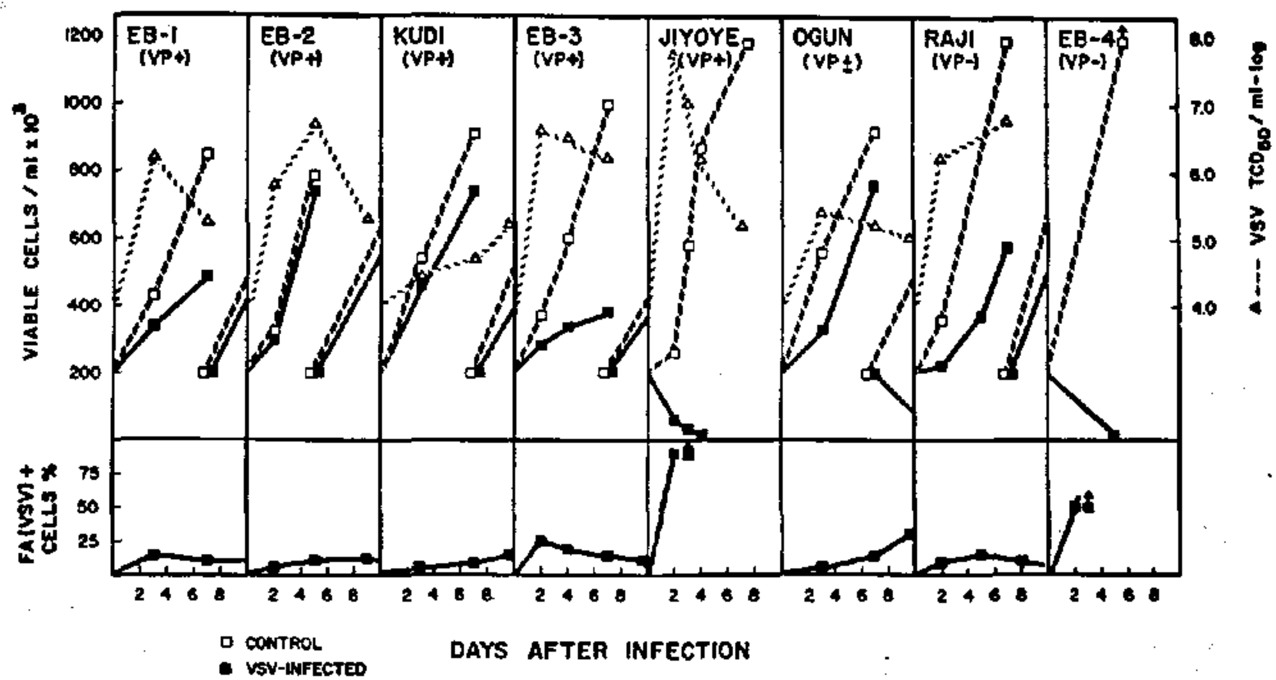
1. Procedure: 5×10^6 cells were sedimented by centrifugation at 1000 rpm for 10 minutes. The medium was decanted and the pellet resuspended in 2.0 ml of maintenance medium containing 1×10^7 PFU per ml of VSV. The cell-virus mixture was incubated, with intermittent agitation for 1 hour at 37 C and then centrifuged. The cells were resuspended in growth medium to a concentration of 2×10^5 per ml. Samples were taken immediately to determine the number of viable cells per ml and the background virus and the cultures were then incubated at 37 C. Uninfected control cultures were handled and maintained in parallel. At varying intervals after exposure to virus, further samples were removed from the cultures for (1) total and viable cell counts; (2) preparation of smears for determination of the number of cells showing VSV-specific

immunofluorescence; and (3) assay of infectious virus. In experiments extending over more than seven days, the cell density of the cultures was reduced to 2 x 10^5 per ml at weekly intervals by dilution of the cell suspension in appropriate amounts of fresh growth medium.

2. Results: Figure 1 represents a composite of the responses of 8 Burkitt cell lines to infection with VSV. Six of these were known to harbor herpes-type virus particles whereas in two virus particles were not demonstrable. Of the 5 cell lines which revealed a marked resistance to VSV four (EB-1, EB-2, EB-3 and Kudi) contained demonstrable herpes-type particles, while the fifth (Raji) was virus-negative. The effect of VSV infection in these five cultures was as follows: (1) cellular growth rates during the first seven days were affected to different degrees. Cell growth was reduced by 5% in EB-2, 20% in Kudi, 40-50% in EB-1 and Raji, and 60% in EB-3 cultures; (2) cells showing virus-specific immunofluorescence in all lines never exceeded 25%; and (3) the peaks of infectious virus, ranging between 10^6 and 10^7 TCD_{50} per ml, were reached within 2-5 days. Of the three Burkitt cell lines that succumbed to VSV infection, two (Jijoye and Ogun) contained herpes-type particles, while the third (EB-4) did not. Jijoye and EB-4 cultures were rapidly destroyed by VSV. The number of Jijoye cells containing VSV antigen as measured by specific immunofluorescence reached 60% on day 1 and 100% on day 3. Peak infectivity titers, approximately 10^8 TCD_{50} per ml, were reached within 2 days. Ogun cells were judged somewhat more resistant since the culture succumbed only by the 10th day.

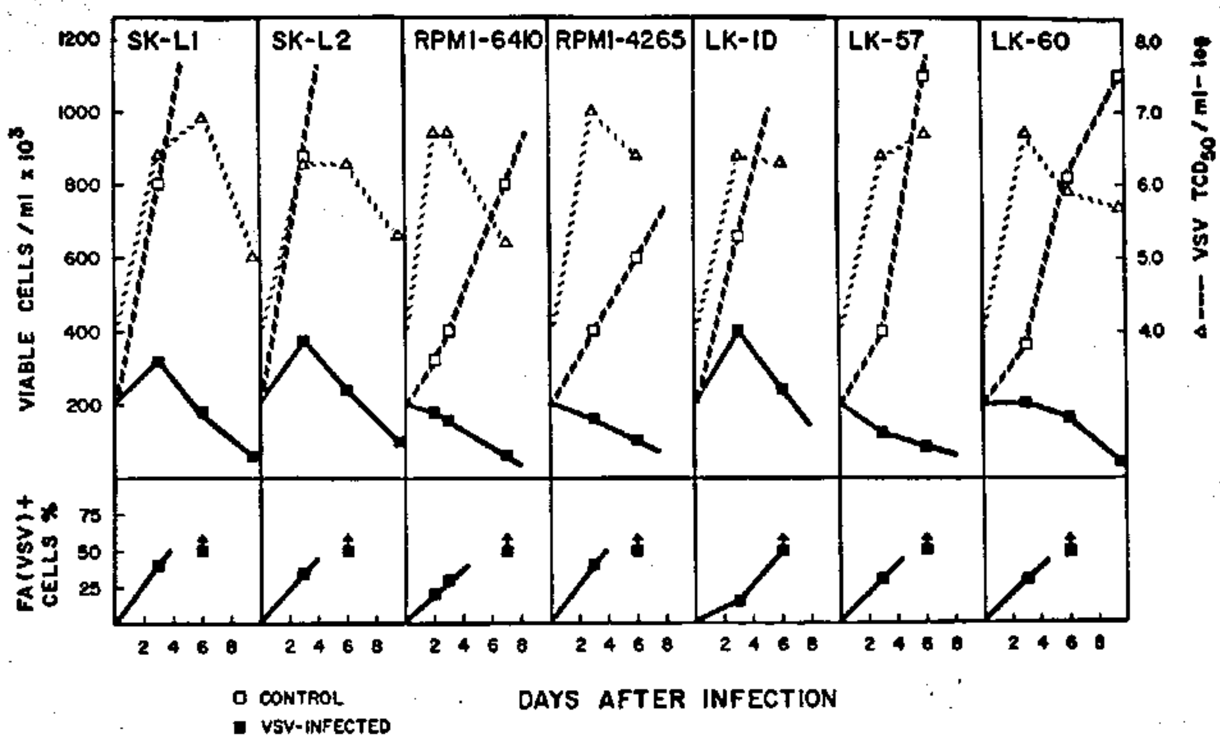
Figure 2 represents a composite of the responses to VSV of seven cell lines derived from patients with leukemia. The VSV infections were characterized, in all instances, by early cell death, rapid increases

FIGURE 1



EB-1 (VP+)
EB-2 (VP+)
KUDI (VP+)
EB-3 (VP+)
JIYOYE (VP+)
OGUN (VP±)
RAJI (VP-)
EB-4 (VP-)
VIABLE CELLS / ml x 10^3
1200
1000
800
600
400
200
FA(VSV)+ CELLS %
75
50
25
VSV TCD50 / ml-log
8.0
7.0
6.0
5.0
4.0
2 4 6 8
□ CONTROL
■ VSV-INFECTED
DAYS AFTER INFECTION

FIGURE 2


SK-L1
SK-L2
RPMI-6410
RPMI-4265
LK-ID
LK-57
LK-60
VIABLE CELLS / ml x 10³
1200
1000
800
600
400
200
△---- VSV TCD50 / ml - log
8.0
7.0
6.0
5.0
4.0
FA(VSV)+ CELLS %
75
50
25
2 4 6 8
DAYS AFTER INFECTION
□ CONTROL
■ VSV-INFECTED

in cells showing virus-specific immunofluorescence and in infectious virus titers. None of these lines survived the VSV infection.

Burkitt cell lines which were found significantly resistant to VSV were maintained for various intervals after infection in order to determine whether or not the virus persisted in the cultures. In earlier studies (Henle and Henle, 1965a, b), the EB-1 and EB-2 lines were maintained for four weeks after infection. Reduction in cellular growth rates fluctuated between 5 and 50% during this period. The percentage of cells showing virus-specific immunofluorescence varied from 5-15%, and VSV-infectivity titers remained at levels above 10^5 TCD_{50} per ml. These observations were confirmed and extended with additional Burkitt lymphoma cell lines showing a marked degree of resistance to VSV. Figure 3 demonstrates the course of VSV infection in Raji cultures over a 41 day period. The cellular growth rates were reduced throughout the period of observation as compared to those of uninfected control cultures. The reduction in growth rates ranged from 38 to 70% at the various times of assay. The concentration of infectious VSV increased from $10^{4.2}$ TCD_{50} per ml immediately after exposure to $10^{6.2}$ TCD_{50} per ml in two days, at which level it remained constant for the duration of the experiment. Virus-specific immunofluorescence revealed 6% of the cells infected by the second day and from 10-15% in the following 39 days. The yields of virus per infected (fluorescent) cell were 60 TCD_{50} at 2 days, but thereafter declined rapidly to values close to 1 TCD_{50} per cell.

Figure 4 represents the course of VSV infection in EB-3 cultures maintained for 42 days. The cellular growth rates were reduced thoughout the period of observation as compared to the uninfected cells.

FIGURE 3

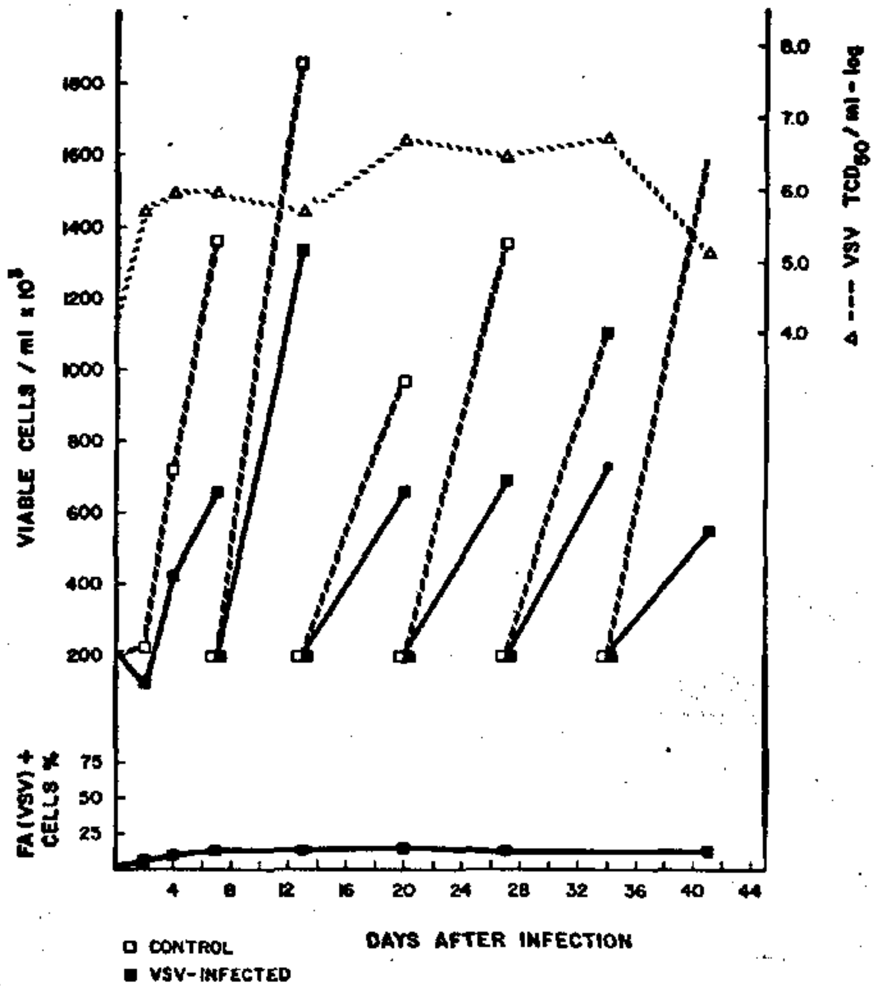
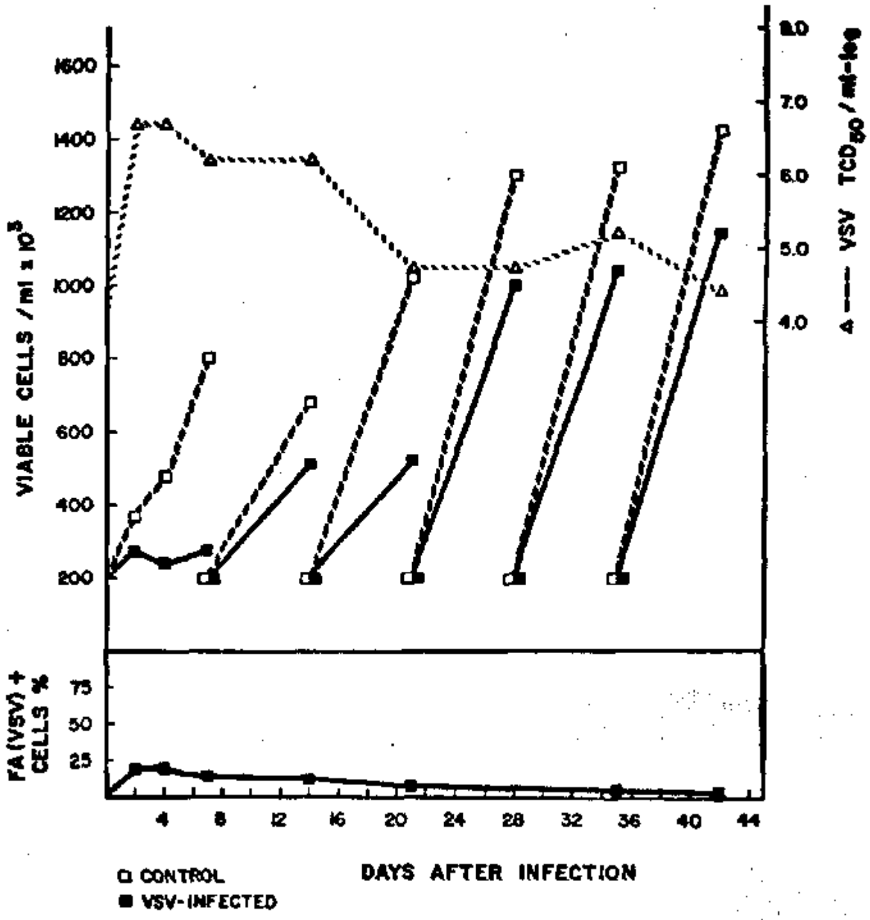

VIABLE CELLS / ml x 10³
1800
1600
1400
1200
1000
800
600
400
200
FA (VSV) + CELLS %
75
50
25
8.0
7.0
6.0
5.0
4.0
Δ ---- VSV TCD50 / ml - log
4
8
12
16
20
24
28
32
36
40
44
DAYS AFTER INFECTION
□ CONTROL
■ VSV-INFECTED

FIGURE 4


VIABLE CELLS / ml x 10^3
1600
1400
1200
1000
800
600
400
200
VSV TCD_{50} / ml-log
8.0
7.0
6.0
5.0
4.0
FA (VSV) + CELLS %
75
50
25
4
8
12
16
20
24
28
32
36
40
44
DAYS AFTER INFECTION
CONTROL
VSV-INFECTED

During the first seven days, the reduction in cell number amounted to 65%, but thereafter a gradual increase in the growth rate was noted which was accompanied by decreases in virus-specific immunofluorescence and infectivity titers of VSV. The concentration of virus increased from $10^{4.2}$ TCD_{50} per ml to about $10^{6.7}$ TCD_{50} per ml on day 2, and decreased gradually thereafter. Virus-specific immunofluorescence showed 20% of the cells to be infected by the second day and decreased to less than 5% in the follwoing 42 days. The yields of virus per infected (fluorescent) cell were 90 TCD_{50} on the second day but declined to values of less than 1 TCD_{50} by the 42^{nd} day.

A summary of the responses of all the cell lines to infection with VSV is shown in Table II. Also included in the table are the responses of the cells to herpes simplex virus (HSV) (Henle *et al.*, unpublished). The first five Burkitt lymphoma cell lines listed were significantly resistant to infection with VSV and HSV. Resistance to VSV resulted in the establishment of a VSV carrier state. The resistance to HSV was characterized by survival of the cultures for 3 to 7 weeks, during which time cells showing HSV-specific immunofluorescence gradually rose from $<$ 1% to $>$ 50%. Only the Kudi cell line recovered from the herpes infection. Jijoye and Ogun cultures succumbed to both VSV and HSV. The Raji line became a carrier of VSV but succumbed to HSV, whereas the EB-4 cells were resistant to HSV but not VSV. All cell lines derived from leukemic patients were completely destroyed when exposed to either VSV or HSV.

TABLE II

Resistance of various cell lines to infection with vesicular stomatitis and herpes simplex viruses

Origin	Cell line	Resistance to VSV	Resistance to HSV(1)
Burkitt tumor	EB-1	+C(2)	+D(3)
	EB-2	+C	+D
	SL_1	+C	n.d.(4)
	Kudi	+C	+R(5)
	EB-3	+3	+D
	Jijoye	-	-
	Ogun	-	-
	Raji	+C	-
	EB-4	-	+D
Leukemia	SK-L1	-	-
	SK-L2	-	-
	6410	-	-
	4265	-	-
	LK-1D	-	-
	LK-57	-	-
	LK-60	-	-

(1) Henle et al., unpublished
(2) VSV carrier states in the surviving cultures
(3) Death of the culture was delayed by 3-7 weeks
(4) Not done
(5) Culture recovered

It was evident that the cell lines tested were affected by VSV infection to different degrees as demonstrated by cellular growth, the percentages of cells showing VSV-specific immunofluorescence, and infectious VSV titers. Those among the Burkitt cell lines which were relatively resistant to VSV became chronically infected and presumably could have been maintained as carrier cultures for much longer periods of time than tested, if not indefinitely.

B. <u>Transfer of resistance to other human cells on mixed cultivation</u>: It has been demonstrated by Deinhardt et al. (1958), that transfer of L_{NDV} populations to test cultures of normal L cells established resistance to VSV in the recipient populations within 24 hours. The development of resistance was attributed to the transfer of (a) non-infectious, interferring NDV particles; (b) interferon released from transferred cells; and (c) production of interferon by cells of the recipient cultures resulting from exposure to NDV particles produced by carrier culture cells (Rodriguez and Henle, 1964). Preliminary experiments (Henle and Henle, 1965a, b) have shown that the resistance to VSV of Burkitt lymphoma lines (EB-1 or EB-2) could be transferred by intact, but not disintegrated cells to recipient cultures of human, and lower animal cells. These studies were extended to other Burkitt and leukemia cell lines.

1. <u>Procedures</u>: The cells to be tested were counted, sedimented by centrifugation at 1000 repm for 10 minutes, and resuspended in a medium consisting of REMX (47.5%) HMEM (47.5%), FCS (5%) to yield 1.0 x 10^6 cells per ml. Two-fold dilutions of the cells were then made in the above medium. Recipient tube cultures of various types of cells were first drained of medium and then seeded with 1.0 x, 0.5 x, or 0.125 x 10^6

test cells in one ml volumes. The tubes were incubated at 37 C. Control monolayer cultures, without test cells, were carried in parallel. After 24 hours of incubation, a portion of the test and control cultures were challenged with 100-1000 TCD_{50} of VSV. The remainder of the cultures were challenged with VSV 3 to 14 days later. The results were recorded when the controls showed complete destruction of the monolayers.

2. Results: Table III demonstrates the ability of EB-1 and EB-2 cells to transfer resistance to human embryonic kidney (HEK) and green monkey kidney (GMK) recipient cultures, respectively. It can be seen from these examples that when 1.0×10^6 cells were seeded onto the monolayers, the recipient cultures became resistant to VSV within 24 hours. With a decrease in the number of lymphoma cells transferred, resistance developed with an increasing delay. Once resistance to VSV challenge had been established, the recipient cultures remained protected as long as both types of cells survived. Exposure of rabbit and murine cells to lymphoma cells, even for extended periods of time, did not alter their susceptibility to VSV challenge.

Table IV summarizes the results obtained with other cell lines used to confer protection against VSV in recipient monolayers. Four of nine Burkitt lines tested were able to induce resistance to VSV in recipient HEK cultures within 24 hours. With Jijoye cells more than 24 hours' contact was required before protection could be measured in recipient cultures. Kudi, Raji, EB-3 and EB-4 cells did not confer resistance at any time. Five of six leukemia lines, two lines from healthy donors, and three of fourteen preparations of fresh leucocytes were capable of protecting HEK cultures. The ability of 12 of the 17 lines tested

TABLE III

Demonstration of resistance to VSV in recipient cultures following transfer of decreasing numbers of Burkitt lymphoma cells

Cells transferred	Recipient culture	Day of VSV challenge	Number of cells transferred (x 10^6)				
			1.0	0.5	0.25	0.125	none
EB-1	HEK	1	0[1]	<1	2	3	4
		7	0	0	0	0	4
		14	0	0	0	0	4
EB-2	GMK	1	0	2	4	n.d.[2]	4
		3	0	±	<1	n.d.	4
		5	0	0	±	n.d.	4
	RK	1	4				4
		7	4				4
		14	4				4
		21	4				4
	L(MCN)	1	4				4
		5	4				4
		18	4				4

(1) CPE produced by VSV 2-3 days after challenge, 0-4+ from no to complete destruction.
(2) Not done

TABLE IV

Transfer of resistance to recipient HEK cultures by various cell lines

Origin	Cell line	Transfer of resistance (1×10^6 cells)	
		VSV challenge Day 1	VSV challenge Day 5-7
Burkitt tumor	EB-1	+	+
	EB-2	+	+
	SL_1	+	+
	Kudi	-	-
	EB-3	-	-
	Jijoye	-	+
	Ogun	+	+
	Raji	-	-
	EB-4	-	-
Leukemia	SK-L1	+	+
	SK-L2	+	+
	6410	-	-
	LK-10	+	+
	LK-57	-	+
	LK-60	-	+
Non-leukemic donors	7666	-	+
	7466	+	+
Fresh leucocytes	3 donors	+	n.d.(1)
	11 donors	-	n.d.

(1) Not done

to cause resistance in recipient cell cultures suggested that an inhibitor was released by these cells. The apparent species specific orientation of the transfer effect (Table III) suggested that an interferon was probably responsible for the observed protection.

C. Test for interferon in culture media: The presence of interferon in L_{NDV} carrier cultures (Henle et al., 1959), capable of inducing resistance to VSV challenge in murine cells, but not in cells of unrelated species, suggested that the resistance to VSV conferred by Burkitt lymphoma and leukemia cells to HEK and GMK cultures also might be due to the production of an interferon. To test this possibility the following experiments were carried out.

1. Procedure: The cells of various lines to be tested were counted, centrifuged, and the pellet resuspended in sufficient growth medium to a final cell concentration of either 1×10^6 or 1×10^7 per ml. The suspensions were transferred to flasks and those containing 1×10^6 cells per ml were incubated at 37 C for 6 days; those containing 1×10^7 cells per ml were incubated on a rotray shaker at 37 C for 24 hours. The media were harvested and, following centrifugation at 1500 rpm for 10 minutes, stored at 4 C until assayed for the presence of interferon.

2. Results: Table V shows that four of the Burkitt lines produced an inhibitor, while four did not. Of the Burkitt lines that produced the inhibitor, Ogun cultures consistently produced the greatest amounts. The ability of EB-1 cultures to produce inhibitor varied and a titer of 24 units per ml was the optimum obtained. The other Burkitt cell lines produced no detectable titers. Surprisingly, the cell lines derived from healthy donors and all the leukemia lines, with the exception

TABLE V

Production of an interferon-like inhibitor
by various cell lines

Origin	Cell line	Interferon-like inhibitor units per ml
Burkitt tumor	EB-1	24
	EB-2	12
	SL_1	12
	Kudi	< 2
	EB-3	< 2
	Jijoye	< 2
	Ogun	32
	Raji	< 2
Leukemia	SK-L1	32
	SK-L2	≥ 24
	6410	< 2
	LK-10	≥ 24
	LK-57	≥ 24
	LK-60	≥ 24
Non-leukemic donors	7666	≥ 32
	7466	16

of 6410, produced good yields of the inhibitor. As will be shown in Section II, the inhibitors found fulfill present criteria for an interferon. It appears that some of the cells tested produce interferon without evident stimulation ("autogenous" interferon).

D. Production of interferon on viral stimulation: The low titer of interferon observed in the L_{NDV} carrier population was ascribed to resistance of most of the carrier culture cells to infection, refractory state to induction of interferon synthesis (Cantell and Paucker, 1963a), and the generally low concentrations of NDV which failed to provide optimal stimulation for interferon synthesis (Henle, 1963). Nevertheless, upon extraneous stimulation of carrier populations with non-infectious virus, the carrier culture cells produced additional interferon but only 10-25% of the amount which could be elicited under similar conditions from non-carrier control cultures (Henle, 1963). The resistance to VSV and the production of low titers of interferon observed in some of the Burkitt cell lines suggested that these may behave like L_{NDV} carrier cultures upon stimulation by an extraneous virus and produce additional amounts of interferon. The following experiments were designed to test this possibility and to compare the results with those obtained in non-resistant cell lines which did or did not produce autogenous interferon.

1. Procedure: 1×10^8 cells were sedimented by centrifugation at 1000 rpm for 10 minutes. The medium was decanted and the cell pellet resuspended in 5.0 ml of medium containing ultraviolet inactivated NDV (NDV_{uv}) at an input multiplicity of 50, based on EID_{50} prior to irradiation. Following an adsorption period of one hour at 37 C on a rotary shaker, 5.0 ml of maintenance medium was added and incubation was continued

with shaking for an additional 23 hours at 37 C. Control cultures, treated with maintenance medium only, were carried in parallel. The media were harvested and, following centrifugation at 1500 rpm for 10 minutes, the supernatants were dialyzed for three days at 4 C, against 100 volume of HCl-KCL buffer at pH 2.0 and then for another 24 hours against 100 volumes of phosphate-buffered saline (PBS) at pH 7.0, a procedure shown to inactivate all interferring activity of NDV_{uv} (Cantell and Paucker, 1963a). Some of the interferon preparations were titrated nevertheless both in the presence and absence of anti-NDV serum and yielded, as expected, identical results.

2. Results: The data presented in Table VI demonstrate that all the various cells tested responded to stimulation by NDV_{uv} with production of more interferon. Four of the Burkitt lines (EB-1, EB-2, Ogun, and EB-3) yielded very small quantities (from 8 to 24 units per ml) as compared to Raji or Jijoye cells which synthesized 200 to 500 units per ml. Only 2 of the cell lines derived from leukemic patients, (SK-L1 and 6410) were tested. Both lines produced substantial quantities of interferon. The titers obtained with SK-L1 cells were consistently high (1000 units per ml), whereas the titers produced by 6410 cells varied but never exceeded 300 units per ml. The 10 fresh leucocyte preparations responded well to stimulation with NDV_{uv}, with titers ranging from 64 to 1024 units per ml, in confirmation of Gresser (1961), and Lee and Ozere (1965). While the yields of virus-induced interferon seemed to relate to some extent to autogenous interferon production exceptions were noted: EB-3 cells produced little virus-induced and no autogenous interferon and the SK-L1 line high titers of virus-induced interferon in spite of the fact that it also yielded autogenous inhibitor.

E. Protection by exogenous interferon: The preceding experiments have demonstrated that some of the cell lines derived from Burkitt

TABLE VI

Interferon production by various cell lines when stimulated by NDV_{uv}

Origin	Cell line	Interferon units per ml	
		Control cultures	NDV_{uv}- exposed cultures
Burkitt tumor	EB-1	<2*	16
	EB-2	<2*	16
	EB-3	<2	8
	Ji Joye	<2	192
	Ogun	2*	24
	Raji	<2	512
Leukemia	SK-L1	4*	1024
	6410	<2	128
Fresh leucocytes	10 Donors	<2	64-1024

* The levels of autogenous interferon were lower than those shown in Table V because residual interferon was removed from the test cultures at time of challenge with VSV

lymphomas or leukemic patients produced an interferon-like inhibitor yet these cells were fully susceptible to infection with VSV. To resolve this apparent disparity, experiments were designed to determine whether these cell lines could be protected by an exogenous interferon against challenge with VSV, and whether the VSV-susceptible fraction of resistant lines could likewise be rendered resistant in this manner.

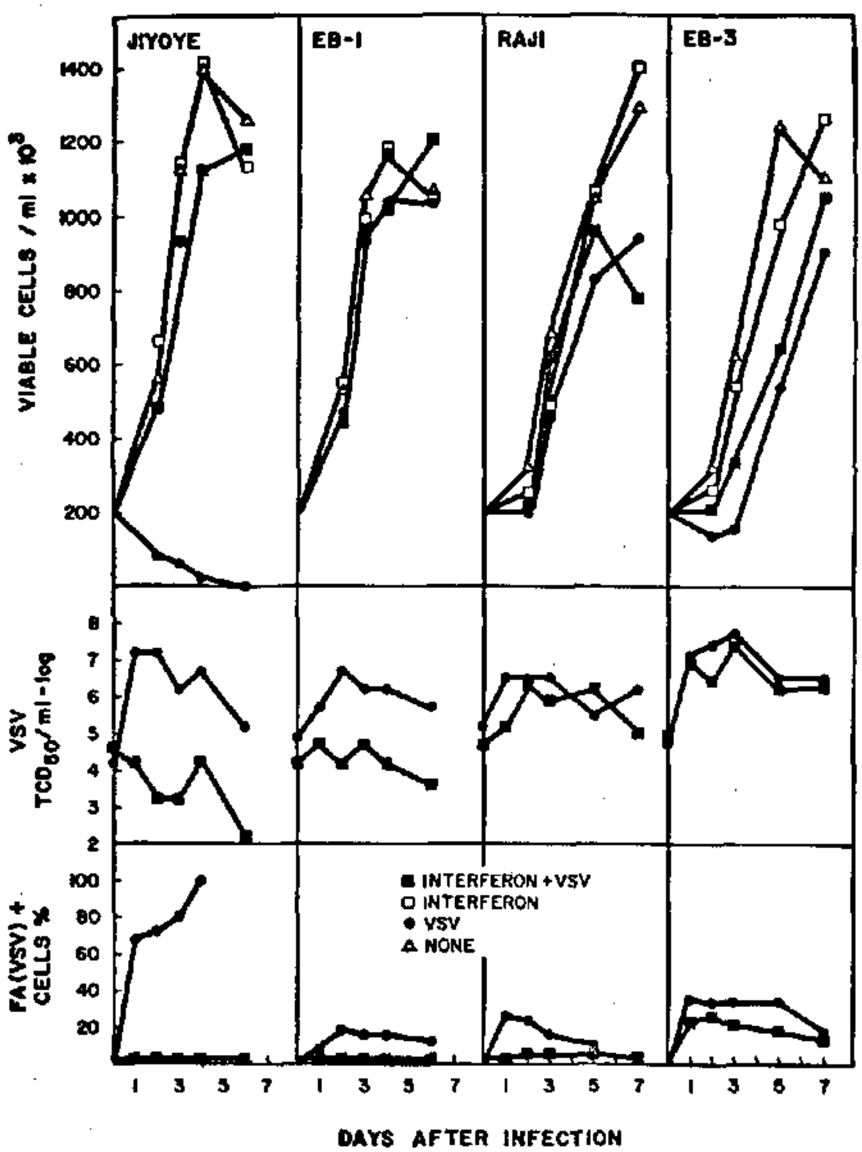
1. Procedure: 1×10^7 cells were centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended in 5.0 ml of interferon (about 200 units per ml) prepared in SK-L1 cells following stimulation with NDV_{uv}. The suspension was transferred to a flask and incubated, with continuous agitation, at 37 C for 3 hours when 10.0 ml of maintenance medium was added. After 24 hours at 37 C the cells were counted and challenged with VSV at an input multiplicity of 2 by the usual method. Virus was permitted to adsorb for 2 hours at 37 C, with continuous agitation. Following adsorption the volume of the culture was adjusted with growth medium to give a cell concentration of 2×10^5 per ml and the final concentration of fetal calf serum was increased to 10%. Control cultures, carried in parallel, consisted of cells treated (a) only with VSV, virus control; (b) only with interferon, interferon control; and (c) only with maintenance medium, cell control. Immediately after final adjustment of the cell concentration samples were taken in order to determine the number of viable cells and background virus. At varying time intervals after exposure to the virus, the cultures were sampled for total and viable cell counts, preparation of cell smears for staining with anti-VSV fluorescein-conjugate, and assay for infectious virus.

2. __Results__: The data shown in Figure 5 are representative of the four types of responses to human interferon seen with the cell lines tested. Jijoye cells exposed to a potent human interferon for 24 hours showed a solid resistance to challenge with VSV. Infection of the control cultures led to death of the cells in 3 days, an increase in the number of cells showing virus-specific immunofluorescence to 70% on day 1, and yields of virus as high as $10^{7.2}$ TCD_{50} per ml in 24 hours. In contrast, the cells exposed to interferon grew at an only slightly decreased rate as compared to the uninfected controls, rarely contained VSV-specific antigen, and released little, if any infectious VSV progeny. The decline in infectious virus titer was not unlike the rate of VSV inactivation at 37 C.

EB-1 cells exposed to human interferon also showed a markedly increased resistance to challenge with VSV. The infection did not significantly affect the rate of growth of either control or interferon-treated EB-1 cells, the decrease never exceeding 15%. The protective effect of the interferon, however, could be observed in virus-specific immunofluorescence and infectivity titers. Virus-specific immunofluorescence revealed that less than 0.01% of the interferon-protected cells contained VSV antigen. This represented a reduction by greater than 95% as compared to the results in the virus control. The VSV titers were throughout 1.5 to 2.5 log units lower than those of the virus control. No increase in virus titer over the background level could be detected in the interferon treated cultures during the 6 days of observation.

Raji cells treated with human interferon showed only a transitory and partial increase in protection against challenge with VSV. The protective effect of interferon was best demonstrated by virus-specific

FIGURE 5


JIYOYE
EB-1
RAJI
EB-3
VIABLE CELLS / ml x 10^3
VSV TCD50/ml-log
FA(VSV) + CELLS %
INTERFERON + VSV
INTERFERON
VSV
NONE
DAYS AFTER INFECTION

immunofluorescence which showed a 94% reduction in stainable cells on day 1, 65% on day 3, but none on day 5. Only during the first 3 days did interferon-treatment reduce the per se limited effect of VSV on the growth rate of control Raji cells. The production of VSV progeny, likewise, was only slightly delayed.

In comparison to the above cell lines, addition of exogenous interferon to EB-3 cells failed to have a significant effect on the course of the VSV infection. The cellular growth rates of the VSV-challenged control and interferon-treated cultures were similar. Cells showing virus-specific immunofluorescence in the interferon group were reduced, on the average, by no more than 25% as compared to the virus control, and the infectivity titers were comparable.

The responses of all the various cell lines tested to human interferon are summarized in Table VII. The EB-1, EB-2, Jijoye, and Ogun cultures could be solidly protected by interferon against VSV challenge. Raji cultures showed a protective effect only during the first 72 hours after challenge. This transitory protection was evident irrespective of whether the concentration of interferon was 60 units or 200 units per ml. In contrast, interferon treatment of EB-3 cultures provided no significant protection against challenge with VSV. Both leukemia lines tested were solidly protected by interferon against challenge with VSV. Their responses were similar to those of Jijoye cultures as depicted in Figure 5.

F. Discussion: The various results obtained in the above studies are summarized for comparison in Table VIII. It became apparent that the previously reported (Henle and Henle, 1965a, b) analogies in the behavior of L_{NDV} carrier cultures and the first three available Burkitt cell lines

TABLE VII

Protection of cells by human interferon against VSV

Origin	Cell line	Protection by human interferon
Burkitt tumor	EB-1	++(1)
	EB-2	++
	EB-3	$\pm$(2)
	Jijoye	++
	Ogun	++
	Raji	+(3)
Leukemia	SK-L1	++
	6410	++

(1) Culture fully protected by human interferon
(2) Doubtful protection
(3) Partial protection

TABLE VIII

Summary of properties of various cell lines*

Origin	Cell line	VP(1)	FA(2)	Resistance to VSV	Resistance to HSV	Transfer of VSV resistance to HEK cells	Interferon-like Inhibitor	Interferon induction by NDV_{uv}	Protection by human interferon
Burkitt tumor	EB-1	+	+	+	+	+	+	± (3)	Complete
	EB-2	+	+	+	+	+	+	±	Complete
	SL_1	+	+	+	n.d.	+	+	n.d.(4)	n.d.
	Kudi	+	+	+	+	-	-	n.d.	n.d.
	EB-3	+	+	+	+	-	-	(±)	Doubtful
	Ji Joye	+	+	-	-	±	-	+(5)	Complete
	Ogun	±	±	-	-	+	+	±	Complete
	Raji	-	-	+	-	-	-	++(6)	Partial
	EB-4	-	-	-	+	-	n.d.	n.d.	n.d.
Leukemia	SK-L1	-(7)	(±)(8)	-	-	+	+	++	Complete
	SK-L2	-	(±)	-	-	+	+	n.d.	n.d.
	LK-10	-	(±)	-	-	+	+	n.d.	n.d.
	LK-57	-	-	-	-	+	+	n.d.	n.d.
	LK-60	-	(±)	-	-	+	+	n.d.	n.d.
	6410	(+)(9)	-	-	-	-	-	+	Complete
	4265	n.d.	-	-	-	-	n.d.	n.d.	n.d.
Non-leukemic donors	7666	+	±	n.d.	n.d.	+	+	n.d.	n.d.
	7466	+	±	n.d.	n.d.	+	+	n.d.	n.d.

*Refer to page xxvii for explanation of table.

(EB-1, EB-2, and SL_1), all harboring herpes-type virus particles, were largely due to chance selection. Extension of these types of analyses to other Burkitt cell lines as well as to continuous cultures of hematopoietic cells derived from leukemic patients and healthy donors revealed remarkable variations in behavior.

The presence of herpes-type virus particles did not necessarily accompany the resistance of the cell cultures to VSV or HSV. Of seven virus-positive Burkitt lines only five were resistant to these agents. Conversely, of two virus-free Burkitt lines only one succumbed to VSV but it was resistant to HSV, and the other was highly susceptible to HSV but not VSV. The cell lines from leukemic patients or healthy donors, some of which undoubtedly were carrying low levels of herpes-type virus, showed no resistance to either challenge agent.

The results of cell transfer to recipient human cultures in all but one instance matched the results of tests for interferon-like inhibitors in the culture media of the corresponding cell lines; i.e., if on mixed cultivation the recipient cells became resistant to VSV, cultures of donor cells were found to produce inhibitor and vice versa. With the exceptional Jijoye line, cell transfer induced resistance in the recipient cells only with some delay and no inhibitor was detected in the culture media. This discrepancy may be due to different sensitivities of the two types of assays which may become evident when very little inhibitor is produced. Because of the general correlation of the results of these two tests, it is likely that the cell transfer effect is entirely due to release of interferon-like inhibitor from the transferred cells. These data will, therefore, be discussed together.

It is evident from Table VIII that resistance of the various types of cultures to VSV or HSV was not necessarily related to the presence of interferon-like inhibitor. Among the resistant lines several were found which were incapable of imparting protection to recipient cells. Conversely, several of the highly susceptible lines from Burkitt tumors, leukemic patients, or healthy donors conferred excellent protection against VSV to recipient cultures and produced correspondingly appreciable amounts of inhibitor. It became evident that some of the continuous cultures of hematopoietic cells were releasing interferon-like substances without evident stimulation. Rabson et al. (1966) noted that the AL1 line of Burkitt cells continued to yield inhibitor after it was spontaneously cured from infection by herpes-type virus. Deinhardt (personal communication) noted that peripheral leucocytes and bone marrow cells protected recipient human cell cultures against VSV due to production of low levels of interferon-like inhibitors. McCombs and Benyesh-Melnick (to be published) made similar observations. Further studies on the autogenous interferon-like inhibitor will be presented in Section II.

The resistance of some of the cell lines also could not be related to the degree of adsorption of VSV. While generally the cell concentrations had to be above 10^7 per ml in order to detect significant adsorption of the virus, no remarkable differences were noted under these conditions between resistant (EB-1) and susceptible (Jijoye) cultures. Using 3×10^7 and 1×10^8 cells per ml, 44% and 70% of the virus, respectively, were adsorbed onto EB-1 cells and 60% and 76% onto Jijoye cells. Adsorption studies with HSV (Henle et al., unpublished) likewise failed to reveal significant differences between the rate and degree of uptake by susceptible and resistant cells.

The induction of interferon synthesis by an extraneous virus (NDV_{uv}), the 4th parameter explored, also yielded different results depending on the cell line employed. All of the cell lines tested produced significant amounts of interferon except the EB-3 line which released, at most, barely detectable amounts. The extent of virus-induced interferon production in 24 hours could not be clearly related to a herpes-type viral carrier state, resistance to VSV or HSV, or to the presence of autogenous interferon. Cell lines free of herpes-type virus were good producers of virus-induced interferon but so was one of the lines which harbored the indigenous agent. Similarly, all but one of the VSV-susceptible cultures synthesized high levels of interferon in response to NDV_{uv} but so did one of the resistant lines. Finally, the presence of autogenous inhibitor did not necessarily prevent production of high titers of virus-induced interferon, and conversely, one cell line free of detectable inhibitor yielded only minimal quantities of interferon when stimulated by NDV_{uv}. As will be shown in Section II the rate of virus-induced interferon production is influenced, however, by autogenous interferon synthesis. The degree of virus-induced interferon production, like the resistance to VSV or HSV, could not be related to significant differences in infection of the various types of cells by NDV. Cell lines which produced moderate amounts of interferon in response to NDV (up to 24 units) revealed in 16 hours after exposure from 10 to 13% of the cells with viral antigens whereas in cultures yielding large amounts of virus-induced interferon (up to 1000 units) 35% of the cells were infected. This difference in the number of cells infected would not seem sufficient to account for the differences in interferon yields.

Addition of exogenous human interferon to the various types of cells, the last parameter studied, was capable of protecting the VSV-susceptible cultures studied and augmented the resistance of the insusceptible lines, with the exception of EB-3 cells. This line, although significantly resistant to VSV, was found to produce no autogenous inhibitor and little, if any virus-induced interferon.

These various observations indicate that cell cultures of similar origins, such as the Burkitt cell lines, composed of morphologically indistinguishable cells, may show a wide range of behavior with respect to resistance to various viruses, interferon synthesis and protection by interferon. Similar observations have been recorded previously. Henle and Henle (1965b) have reported that 3 of 15 lines of human cells transformed in vitro by SV_{40} showed moderate to marked resistance to infection with VSV and poliomyelitis type 2 virus. Cantell and Paucker (1963b) observed that 1 day-old cultures of different sublines of HeLa cells differed in their sensitivities to interferon. Lockart (1965) also noted differences in the protection of several sublines of L cells by interferon and suggested that the genetic constitution of the cells might play a role. It should be noted, however, that L cells carried in different laboratories, were found recently to be infected to varying extents with unidentified viruses (Kindig and Kirsten, 1967). Studies with two lines of polyoma virus-induced hamster tumor cells (Henle and Henle, 1963) have shown that one line could be induced to synthesize interferon and could be protected by interferon, while the other failed on both accounts.

In conclusion it can be stated that the detection of cellular resistance and of interferon-like substances does not offer dependable evidence for viral carrier states in the Burkitt lymphoma and leukemia cell lines. No clear relationship between the properties studied and the presence of the herpes-type virus was indicated. Almost all of the Burkitt lymphoma cell lines had shown individual characteristics which could not be related to the whole as a group. Whatever the ultimate explanation may be for the observed differences, it became obvious that the use of the L_{NDV} carrier state criteria had failed to provide a reliable means for the detection of the herpes-type viral carrier state in Burkitt lymphoma cells.

II. Comparison of Autogenous and Virus-Induced Interferons Produced by Cell Lines Derived from Burkitt's Tumor and Leukemic Patients.

As discussed in the preceding section, it was initially thought that the production of interferon-like inhibitor by some Burkitt lymphoma cell lines was due to infection of the cultures by a herpes-type virus. However, on testing of additional Burkitt and leukemia cell lines, no correlation was found to exist between the presence of virus particles and the production of inhibitor. It became evident that some of the cell lines which did not contain detectable herpes-type particles produced interferon while others with virus particles failed to synthesize detectable amounts of interferon. Apparently some of the cell lines were able to produce interferon without evident stimulation. This inhibitor is referred to as autogenous interferon.

Ho (1964a) suggested the possibility that interferon may be a constituent of the cell, existing partially or entirely preformed. This possibility has been reemphasized as many non-viral substances were shown to be capable of inducing interferon in cell cultures or in animal hosts, indicating that the formation of interferon was a cell directed process.

Several investigators have shown that tissue fluids from uninfected cells occasionally exhibited inhibitory activity to viral action when used as control fluids in interferon titrations. Ho and Enders (1959) noticed that some extracts of normal HeLa cells were inhibitory. Gresser (cf. Ho et al., 1966) noticed that occasionally culture medium of human leucocytes contained an inhibitor. Other investigators also have noticed that extracts of normal mouse brain or serum contained an inhibitor (Vilcek and Stancek, 1963; Postic, cf. Ho et al., 1966; Finter, 1965;

Mendelson and Glasgow, 1966). Whether these inhibitors were autogenous, i.e. interferons which have not been knowingly induced (Ho et al., 1966) or whether they were actually induced in some unknown manner remains unresolved.

Smith and Wagner (1967) using rabbit macrophages have described in detail an in vitro system of interferon production in the absence of known viral infection. These interferon titers could be augmented when the macrophage cultures were incubated in the presence of 10-100 µg per ml of E. coli lipopolysaccharide. Interferon yields from uninfected macrophages amounted to only 1% or less of the yields from NDV-infected macrophages, but the rate of synthesis of spontaneous interferon was similar to that of the virus-induced inhibitor. Studies with actinomycin and puromycin revealed that as in virus-induced interferon synthesis, transcriptional and translational events were required for de novo interferon synthesis by uninfected macrophages. These results indicated that the spontaneous interferon produced by the rabbit macrophages was not a preformed interferon, since the release of preformed interferons in in vivo systems could not be blocked by actinomycin (Ho and Kono, 1965) or puromycin (Youngner et al., 1965).

The production of interferon in vitro by Burkitt lymphoma and leukemia cell lines in the absence of known viral stimulation as well as due to viral stimulation afforded an unique opportunity to study both autogenous and virus-induced interferons produced in one type of cells. The experiments to be presented in this section were designed (1) to determine the optimal conditions for the synthesis of both interferons; (2) to describe the kinetics of interferon synthesis by various cells; and (3) to compare the properties of autogenous and virus-induced interferons.

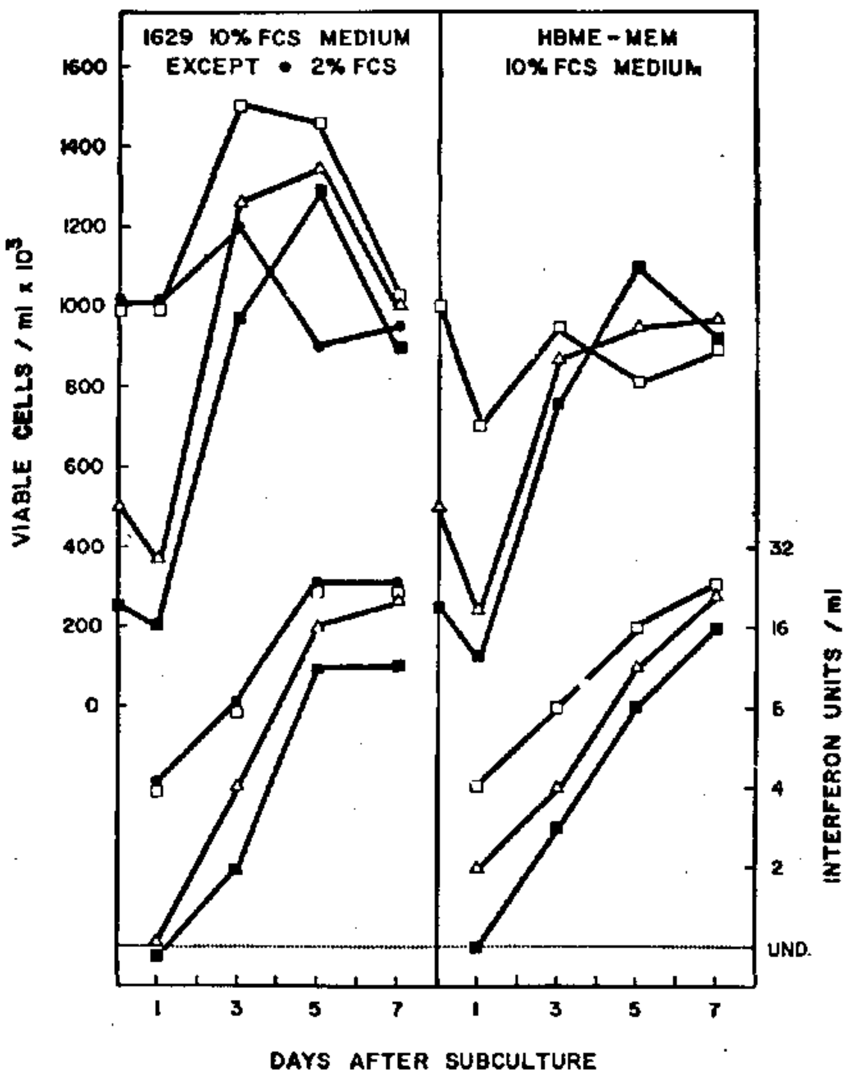
A. <u>Studies on the conditions necessary for the synthesis of autogenous interferon</u>: The small amounts of autogenous interferon released by the cells necessitated the determination of optimal conditions for production of this interferon by Burkitt lymphoma and leukemia cell lines.

1. <u>Effect of cell number, medium and serum concentration</u>:

a. <u>Procedure</u>: Sedimented EB-1 cells were resuspended in sufficient growth medium containing 10% FCS (1629 or HBME-HMEM) to a cell concentration of 1.0 x, 0.5 x, and 0.25 x 10^6 per ml respectively. Additional cells were resuspended in sufficient maintenance medium containing 2% FCS (1629) to a concentration of 1.0 x 10^6 per ml. The suspensions were transferred to individual flasks and incubated at 37 C. On days 1, 3, 5, and 7, the cells were counted and the media harvested, centrifuged at 1500 rpm for 10 minutes and stored at 4 C until assayed.

b. <u>Results</u>: The data presented in Figure 6 demonstrate that the media used to cultivate the cells did not affect the amount of inhibitor produced, since the titers on day 7 were equivalent. It can be seen that the maximal interferon titers were alike but were reached successively later as the initial cell concentration was reduced. An increase in cell number to about 1 x 10^6 per ml was required before maximal titers were reached. Cells grown in medium 1629-2, produced interferon at an equivalent rate as those grown in 1629-10, both ceasing to produce detectable increases of interferon after day 5. These results indicated that the ability of EB-1 cells to produce autogenous interferon was not dependent on the type of medium, or the serum concentration, but that the yield of interferon was dependent on the number of cells per ml.

FIGURE 6


1629 10% FCS MEDIUM
EXCEPT ● 2% FCS
HBME - MEM
10% FCS MEDIUM
VIABLE CELLS / ml x 10^3
1600
1400
1200
1000
800
600
400
200
0
INTERFERON UNITS / ml
32
16
8
4
2
UND.
1
3
5
7
DAYS AFTER SUBCULTURE

2. Variations in cultural techniques: Conditions were sought under which larger amounts of interferon could be produced more rapidly. Experiments were designed to determine the effects of (a) increased cell number, and (b) stationary incubation versus continuous shaking of the cultures on interferon production. Ogun cells were used as a source of autogenous interferon since these cells were found in earlier experiments to be more consistent producers of autogenous interferon as compared to EB-1 cells.

a. Procedure: Ogun cells were counted, centrifuged and the pellet resuspended in sufficient maintenance medium (1629-2) to give 1 x, 5 x, and 10 x 10^6 cells per ml. The cultures containing 1 x 10^6 cells per ml were kept stationary during incubation and harvested at 1, 2, 3, 5, 6, and 7 day intervals. The cultures containing 10 x 10^6 or 5 x 10^6 cells per ml were divided into two parts each, one part was incubated stationary and the other on a rotary shaker. The media were harvested after 24 and 48 hours incubation at 37 C, respectively.

In further experiments a culture containing 1 x 10^7 cells per ml was centrifuged after the first 24 hours of incubation. The cells were resuspended in the original volume of fresh medium and reincubated for an additional 24 hours. This procedure was repeated once more. All media were centrifuged at 1500 rpm for 10 minutes and stored at 4 C until assayed for autogenous interferon.

b. Results: Table IX shows the results of a number of representative experiments. With 1 x 10^6 cells per ml, the rate of interferon synthesis by Ogun cells was similar to that of EB-1 cells (Fig. 6). The maximal titer was reached by day 5. The cultures containing 5 x 10^6 cells per ml, incubated 48 hours with or without shaking, showed that the

TABLE IX

Effect of cell concentration and method of incubation on the yield of autogenous interferon by Ogun cells

Number of cells/ml	Incubation		Autogenous interferon units per ml
	Time	Method	
1×10^6	1 day	Stationary	1
	2 day	"	4
	3 day	"	6
	5 day	"	24
	6 day	"	24
	7 day	"	24
5×10^6	48 hr	Stationary	24
	48 hr	Shaking	32
10×10^6	24 hr	Stationary	16
	24 hr	Shaking	32
	1st 24 hr (1)	Shaking	32
	2nd 24 hr (2)	"	24
	3rd 24 hr	"	16

(1) Cells were centrifuged supernatant saved, pellet resuspended in fresh medium and suspension incubated an additional 24 hours.
(2) Procedure outlined in (1) was repeated.

agitated cultures produced more interferon than the stationary cultures. The cultures containing 1 x 10^7 cells per ml produced 32 units of interferon per ml within 24 hours of incubation on a rotary shaker as compared to 16 units produced by the stationary cultures. Incubation for longer than 24 hours did not increase the titers. It became evident, however, that the same cells on resuspension in fresh medium continued to yield in each 24 hour-interval considerable, though slightly diminishing amounts of interferon probably on account of diminishing numbers of viable cells. This procedure permitted the reuse of cells and provided the most economical and efficient method for obtaining large quantities of autogenous interferon.

B. <u>Studies on the conditions necessary for the synthesis of viral-induced interferon</u>:

1. <u>Screening of different viruses for the capacity to induce interferon synthesis</u>: Two leukemia and one Burkitt lymphoma cell lines were used to study the effect of selected viruses on induction of interferon in these cells. Leukemia cell line SK-LI produced an autogenous as well as virus-induced interferon, while the 6410 leukemia cell line and the Raji Burkitt lymphoma line produced interferon only on virus induction.

a. <u>Procedure</u>: Suspenions containing 1 x 10^7 and 1 x 10^8 cells were centrifuged at 1000 rpm for 10 minutes and resuspended in 1.0 ml of virus. The multiplicities of viable NDV and Sendai virus were based on EID_{50} per ml; of NDV_{uv} on EID_{50} prior to irradiation; of Sindbis virus on PFU per ml in monolayer cultures of chick embryo fibroblasts; of the influenza viruses on HAU per ml taking 1 HAU to be equal to 10^6 infectious units; of vaccinia virus on TCD_{50} per ml in HEK cultures; and of the encephalitis viruses on LD_{50} per ml in mice injected intracerebtally.

The viruses were allowed to adsorb for one hour at 37 C with constant agitation. Following adsorption the cells exposed to viable viruses were centrifuged at 1500 rpm for 10 minutes and the pellets re-suspended in 10.0 ml of maintenance medium. Nine ml of maintenance medium were added to the cells suspended in 1.0 ml of NDV_{uv}. The cell suspensions were transferred to flask and incubated at 37 C for 24 hours. All Raji cell cultures were incubated with constant shaking. The cells were then counted and the media harvested and centrifuged at 1500 rpm for 10 minutes. The supernates were dialyzed at 4 C for 24 hours against 100 volumes of pH 2 buffer and again at 4 C for 24 hours against 100 volumes of PBS. The dialyzed materials were stored at 4 C and assayed for the presence of virus-induced interferon.

b. Results: The data presented in Table X clearly show that only NDV produced measurable quantites of interferon. Both viable NDV and NDV_{uv} induced comparable amounts of interferon in the leukemia lines when used at input multiplicites of 5 and 10 for viable virus, and 5, 10, and 50 for UV-inactivated virus. Although both viable and UV-inactivated NDV stimulated interferon synthesis in Raji cells, it appeared that for viable virus an input multiplicity of at least 5 was required before interferon synthesis could be detected. With 6410 cells measurable interferon synthesis could be induced with an input multiplicity of 1.

2. Effect of cell number, method of incubation and input multiplicities of NDV and NDV_{uv}: The results from the experiments designed to obtain autogenous interferon suggested that similar cultural techniques might be useful for the production of virus-induced interferon. Experiments were designed therefore to investigate the effect of increase in cell number and method of incubation on the pro-

TABLE X

Interferon-inducing capacity of different viruses

Cells	Virus	Input Multiplicity	Interferon units per ml
6410 (1×10^6/ml)	NDV	1, 50, 150,	16
		5, 10	32
	NDV_{uv}	10, 50,	32
		100, 150	12
	Sendai	1, 5, 10, 50	< 4
	Sindbis	1, 5, 10	< 4
SK-41 (1×10^6/ml)	NDV	5	32
	NDV_{uv}	5, 10, 50,	32
		100, 150	16
	Sendai	1,	4
		5, 10, 50	< 4
Raji (10×10^6/ml)	NDV	1,	< 4
		5	12
	NDV_{uv}	50	96
	Influenza A PR8 strain	1	< 8
	Influenza A_2 2496	1	< 8
	Influenza B Johannesburg	1	< 8
	Vaccinia	<1	< 8
	St. Louis encephalitis	1	< 4
	California encephalitis	<1	< 4

duction of virus-induced interferon. Additional experiments were carried out to compare viable and UV-irradiated NDV as interferon inducers since the experiments in the preceding section did not clarify whether or not these preparations possessed equivalent inducing capacities in Burkitt lymphoma and leukemia cell lines.

a. Procedures: Aliquots of cell suspensions containing 1×10^8 cells were centrifuged at 1000 rpm for 10 minutes. The pellets were resuspended in 5.0 ml of medium containing a given multiplicity of NDV_{uv}, or in 1.0 ml of medium containing a given multiplicity of viable NDV. The virus was adsorbed for one hour at 37 C with constant agitation. Following adsorption the cell concentrations were adjusted to 1×10^7 per ml by adding 5.0 ml of maintenance medium (NDV_{uv} series), or the suspension was centrifuged and the pellet resuspended in 10.0 ml of maintenance medium (viable NDV series). The NDV_{uv} cultures were incubated either stationary or on a shaker, while the viable NDV cultures were incubated only with shaking. After 24 hours incubation at 37 C the media were harvested, centrifuged, dialyzed, and stored at 4 C until assayed.

b. Results: As can be seen in Table XI, shaking of the cells during incubation consistently yielded three-to eight-fold increases in interferon yields as compared to the amounts obtained from stationary cells.

The data in Table XII demonstrate that the quantity of interferon produced was dependent upon the cell-virus ratio for both viable and ultraviolet-irradiated NDV. With increasing amounts of viable NDV both SK-L1 and Raji cultures produced increasing amounts of interferon. Viable NDV at an input multiplicity of one failed to induce interferon synthesis in Raji cells, whereas SK-L1 cells synthesized considerable amounts of interferon under this condition. The effect of NDV_{uv} on SK-L1

TABLE XI

Effect of the method of incubation on interferon production by various cell lines induced by NDV_{uv} (i.m. 50)

Cells 1×10^7 per ml	Interferon units per ml			
	Experiment #1		Experiment #2	
	Stationary	Shaking	Stationary	Shaking
SK-L1	192	512	96	256
Raji	8	64	< 8	48
Jijoye	32	96	64	512
6410	16	64	16	64

TABLE XII

Effect of different input multiplicities of viable and inactive NDV on interferon induction

Cell line	Experiment number	i.m. viable NDV	Interferon units/ml	i.m. NDV_{UV}	Interferon units/ml
SK-L1	1	1, 5	192	50	1024
	2	1	128	50	512
	3	1	96	1	192
		5	384	5	512
		10, 50	512	10	512
Raji	1	1	< 8	50	512
	2	1	< 4	1	< 8
		5	12	5	< 8
		10	32	10	< 8
		50	96	50	96

cells appeared to be less dependent on input multiplicity since input multiplicities of 5, 10, and 50 produced equivalent amounts of interferon. In contrast Raji cells could not be induced with NDV_{uv} to yield detectable interferon until an input multiplicity of 50 was reached. Jijoye, 6410, Ogun, and EB-1 cells all responded to viable NDV at input multiplicities of 1 and to NDV_{uv} at input multiplicities of 50. These results suggested that the conditions were similar for induction of interferon synthesis among leukemia and Burkitt cell lines, with the possible exception of Raji cells which required somewhat greater input multiplicites of virus.

The optimal and most dependable condition for stimulation of interferon synthesis was found to be the exposure of the cells at an input multiplicity of 50. Viable NDV was judged less suitable as an inducer since it was more cytolytic than NDV_{uv}.

C. Comparison of kinetics of synthesis of autogenous and virus-induced interferons: Several workers (Isaacs and Burke, 1958; Vilcek and Rada, 1962, Cantell and Paucker, 1963b; Lockart, 1963; Friedman, 1966) have shown that pretreatment of cells with interferon may markedly influence the subsequent ability of cells to produce interferon. Isaacs and Burke (1958) found that pretreatment with interferon enhanced formation of interferon, while Vilcek and Rada (1962) and Cantell and Paucker (1963b) found that pretreatment inhibited yields of interferon. Recently Lockart (1963) and Friedman (1966) showed that pretreatment with crude or semipurified interferon preparations may indeed have both effects depending on the amount of interferon used initially and the dose of active or inactive virus employed subsequently to elicit interferon production.

The experiments presented in the preceding section have indicated that active autogenous interferon production by SK-L1, Ogun, and EB-1 cultures did not prevent these cells to respond to virus induction with additional interferon synthesis, although Ogun and EB-1 cells yielded much lower quantities of virus-induced interferon in 24 hours as compared to those obtained in SK-L1 populations. It was of interest to determine what effect the presence of autogenous interferon would have upon the kinetics of synthesis of virus-induced interferon. Experiments were designed to compare the kinetics of production of autogenous and virus-induced interferons as well as to compare the time of appearance and of synthesis of virus-induced interferon by the different cell lines.

1. Procedure: For comparison of the kinetics of synthesis of autogenous and virus-induced interferons in the same culture, both SK-L1 and Ogun cells were used. The experiments designed to compare the kinetics of synthesis of virus-induced interferon by various lines were carried out with Raji, Jijoye and 6410 cells, representing cultures with no detectable autogenous interferon, and with SK-L1 and EB-1 cells, representing cultures producing autogenous interferon.

a. Autogenous interferon: The cells were counted, centrifuged at 1000 rpm for 10 minutes, and resuspended in maintenance medium to give 1×10^7 cells per ml. The cell suspensions were transferred to flasks and incubated at 37 C on a rotary shaker. Ten ml aliquots of cell suspension were removed after varying intervals of incubation and assayed for autogenous interferon.

b. Virus-induced interferon: The cells were counted, centrifuged at 1000 rpm for 10 minutes, and resuspended in NDV_{uv} (input multiplicity of about 50) to yield 2×10^7 cells per ml. Following adsorption for one hour at 37 C the cell concentration was adjusted with maintenance medium to 1×10^7 per ml. Ten ml aliquots of cell suspension were removed after varying intervals of incubation and processed for interferon assay as described earlier.

2. Results: Figure 7 demonstrates the difference in the rates of autogenous and virus-induced interferon synthesis in SK-L1 cells. Autogenous interferon was detected as early as one hour after incubation at 37 C but did not reach peak concentration before 16 hours. Production of virus-induced interferon likewise became detectable between one and two hours following addition of NDV_{uv} but attained a near maximal level of 1024 units within 8 hours. Titers at 16 hours were approximately twice those seen at 8 hours.

Ogun cells produced autogenous interferon at the same rate and to the same extent as SK-L1 cells. The yields of virus-induced interferon were, however, much lower in Ogun (64 units) than in SK-L1 cells. Nevertheless synthesis of virus-induced interferon in Ogun cells was similarly rapid as in SK-L1 cells.

Figure 8 compares the rates of interferon synthesis induced by NDV_{uv} in SK-L1 and Raji cultures. The data clearly show that the cells differed greatly in their ability to produce virus-induced interferon. No interferon became detectable in Raji cultures within 8 hours of incubation following the addition of NDV_{uv}. Peak levels of interferon were obtained between 17 and 24 hours. In contrast SK-L1 cells produced nearly maximal amounts of interferon by the 6th hour.

FIGURE 7

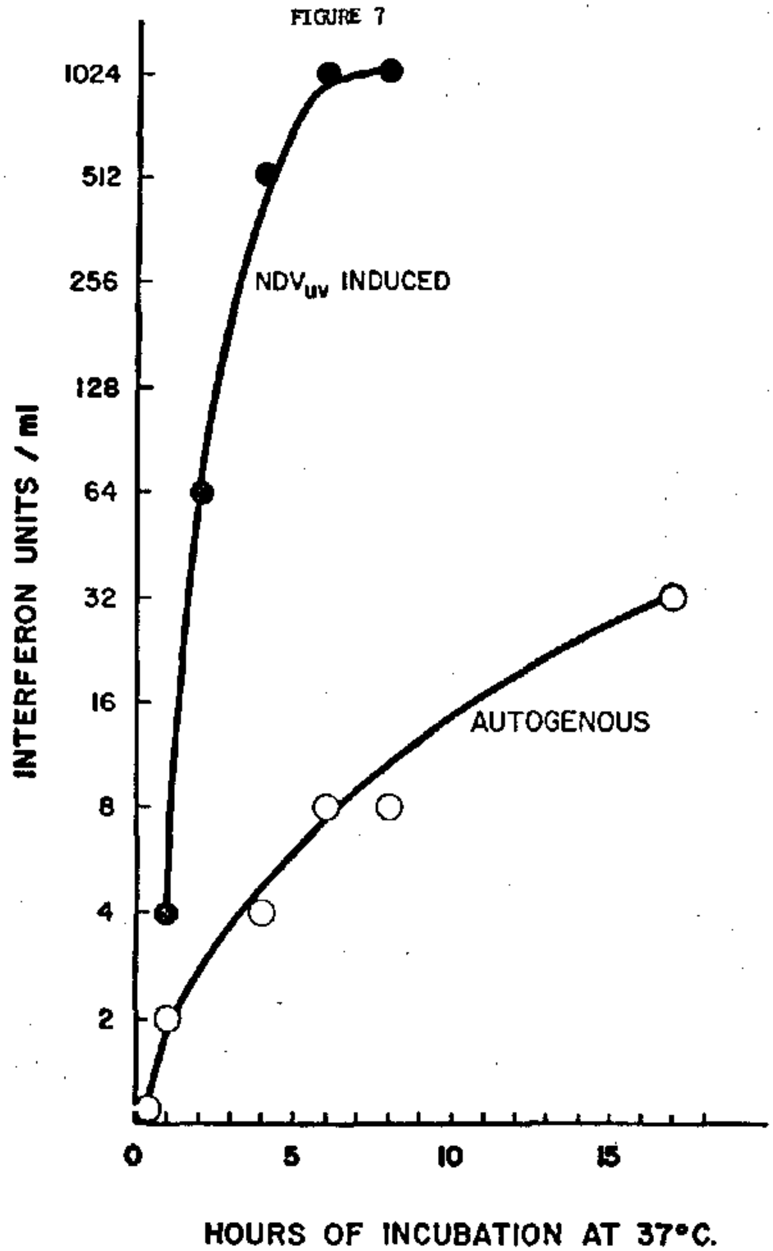
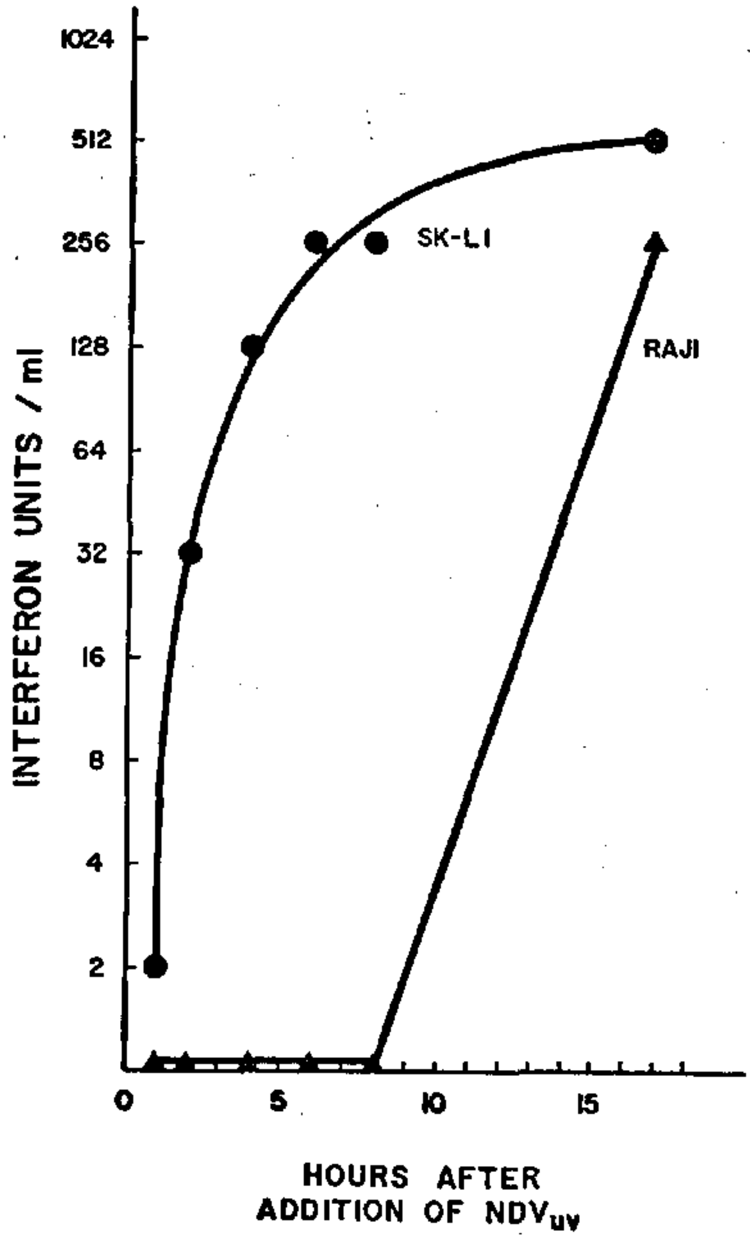

1024
512
256
128
64
32
16
8
4
2
INTERFERON UNITS / ml
NDV$_{UV}$ INDUCED
AUTOGENOUS
0
5
10
15
HOURS OF INCUBATION AT 37°C.

FIGURE 8



INTERFERON UNITS / ml
1024
512
256
128
64
32
16
8
4
2
SK-L1
RAJI
0
5
10
15
HOURS AFTER
ADDITION OF NDV_{uv}

The data presented in Table XIII demonstrate that the rate of virus-induced interferon synthesis in the various cells tested appeared to be influenced by the presence of autogenous interferon. It became evident that in cell lines which synthesized high levels of autogenous interferon (SK-L1 and Ogun) the rate of virus-induced interferon synthesis was enhanced. Nearly maximal amounts of virus-induced interferon were reached by the 6th hour of incubation. Those cells lines which did not produce autogenous interferon (6410 and Raji) showed a lag of at least 8 hours before virus-induced interferon could be detected. While those cell lines which produced low levels or levels detectable only by cell transfer (EB-1 and Jijoye, respectively) showed intermediate rates of virus-induced interferon synthesis similar to the kinetics for autogenous production demonstrated by SK-L1 cells (Fig. 7). Interferon was first detected between 1 and 3 hours after exposure to NDV_{uv} but did not reach a peak until after 17 hours of incubation. These rate studies have indicated that the presence of autogenous interferon in various Burkitt lymphoma and leukemia cell lines appeared to enhance the rate of virus-induced interferon synthesis by these cells.

D. Comparison of the properties of autogenous and virus-induced interferons: Comparative experiments were carried out to determine the physiochemical and biological properties of autogenous and virus-induced interferon. The autogenous inhibitor was obtained from EB-1 and Ogun cells; Raji cells were used to obtain virus-induced inhibitor. In addition a leukemia cell line SK-L1 was chosen because of its unique property of producing in good quantities, both autogenous and virus-induced interferons. NDV_{uv}-induced interferon prepared in HEK monolayer cultures was included as representative of human interferon from primary cells.

1. Procedures: The culture fluids containing interferon were concentrated 5-15x as described in Materials and Methods. All interferon

TABLE XIII

Rates of virus - induced interferon synthesis in Burkitt lymphoma and leukemia cell lines

Lines	Autogenous interferon production	Interferon units per ml			
		Hours after addition of NDV_{uv}			
		1	3	6	17
SK-L1	++	2	64	256	512
Ogun	++	<4	12	32	64
EB-1	+	<2	4	24	96
Jijoye	±	<2	8	64	512
6410	-	<2	<2	<2	128
Raji	-	<2	<2	< 2	256

preparations were tested concurrently for various properties:

(a) Acid stability: Undiluted interferons were dialyzed for 72 hours at 4 C against 100 volumes of HCl-KCl buffer at pH 2.0. To adjust the pH of the samples to neutral the interferons were again dialyzed for 24 hours at 4 C against 100 volumes of PBS pH 7.0.

(b) Nonsedimentability: All interferons were diluted in HBSS to contain between 8 and 32 units per ml and spun at 100,000 x g for 2 hours in a Spinco Model L centrifuge using a #40 rotor.

(c) Heat stability: Three ml aliquots of each interferon, diluted in HBSS, were heated for one hour in a water bath maintained at 56 C or 80 C. Following treatment the samples were immediately chilled.

(d) Trypsin sensitivity: 0.8 ml samples of each interferon were mixed with 0.8 ml of a 0.2% solution of 2x crystallized trypsin (Worthington Biochemical Corp., Freehold, New Jersey) in HBSS. As a control of thermal stability additional samples of interferon were diluted in HBSS only. Both sets of preparations were incubated in a 37 C water bath for 1 hour. The action of trypsin was stopped by addition of FCS to a final concentration of 10% and chilling of the samples in an ice bath. Before titration all the samples were diluted four-fold to decrease the possibility of further trypsin action.

(e) Effect of refeeding of cultures prior to challenge: HEK cells were treated for 24 hours with 2-fold dilutions of interferon. At the time of challenge the interferon containing media were removed from one half of the cultures and 1.5 ml of fresh medium was added. The other half of the cultures served as controls. All the cultures were then challenged with VSV.

(f) Species specificity: Monolayer cultures of murine L(MCN) cells were incubated for 24 hours in the presence of 8 to 32 units of human interferon. After 24 hours of incubation the tubes were challenged with VSV.

(g) Direct anti-viral activity: 100 to 1000 TCD_{50} of VSV were mixed with the various interferons and incubated for one hour at 37 C. The samples were then titrated in HEK cultures for virus infectivity.

2. Results: In Table XIV appears a summary of the physical and biological properties of the interferons. It became evident that the criteria for classification as interferon (Isaacs, 1963) were met by the autogenous as well as the virus-induced interferons derived from the Burkitt lymphoma and leukemia cell lines. The interferons were found to be stable at pH 2.0. All the interferons, with the exception of those produced by SK-L1 cells, showed a 2-to 8-fold reduction in activity after one hour at 56 C. The most labile was the interferon produced in HEK cells. Heating of the interferons at 80 C resulted in complete loss of activity. Trypsin at 0.1% final concentration inactivated all interferon activity in 1 hour at 37 C. None of the interferons protected L(MCN) cells, thus showing species specific orientation. None inactivated VSV *in vitro*. A 3-to 5-fold decrease in titers was obtained when the interferons were removed from the assay cells prior to challenge. This was evident with virus-induced as well as autogenous interferons. It was evident that the autogenous and virus-induced interferon did not differ significantly in their properties. Thus, no basis was found for distinguishing between them by the criteria employed.

TABLE XIV

Properties of autogenous and virus-induced interferons

Interferon treatment	Assay cells	Interferon units per ml					
		Autogenous			Virus-Induced		
		Cell line			Cell line		
		EB-1	Ogun	SK-L1	SK-L1	Raji	HEK
None	HEK	128	128	512	-	-	-
pH 2.0	"	128	96	512	2048	512	256
100,000 x g 2 hr	"	128	128	512	2048	512	256
56 C 1 hr	"	64	96	512	2048	256	32
80 C 1 hr	"	< 4	< 16	< 16	< 64	< 32	< 32
0.1% trypsin 1 hr 37 C	"	< 8	< 16	< 16	< 64	< 32	< 32
Removed(1)	"	20	128	6	256	2048	192
Not removed(2)	"	80	384	32	768	8192	512
None	L(MCN)	< 2	< 8	< 16	< 32	< 16	< 16

(1) Interferon was removed from assay cells prior to challenge.
(2) Interferon remained on assay cells during challenge.

E. Protective effect of autogenous interferon: The results in the preceding section have shown that the autogenous interferons produced by EB-1, Ogun, and SK-L1 cells fulfilled the criteria necessary to characterize them as interferons. Earlier experiments have shown, however, that the Ogun and SK-L1 cultures which yielded autogenous interferon were nevertheless highly susceptible to VSV. This paradox might possibly be explainable by the assumption that too little autogenous interferon was produced to protect a significant proportion of the cells. It became essential, therefore, to determine whether addition of concentrated autogenous interferons produced by these cells could protect them against challenge with VSV.

1. Procedure: Autogenous interferons were collected from Ogun and SK-L1 cells and the culture fluids were concentrated 15 fold prior to use. Virus-induced interferon was prepared for comparison in SK-L1 cells stimulated by NDV_{uv}. Approximately 50-100 units per ml of the autogenous, and 170-200 units per ml of the virus-induced interferon were used to treat the cells.

The cells were centrifuged, resuspended in the proper interferon preparation, and challenged 24 hours later with VSV at an input multiplicity of 2. After an adsorption period of 2 hours the volumes of the cultures were adjusted to yield 2 x 10^5 cells per ml. To control cultures, carried in parallel, were added (a) only VSV; (b) only interferon; and (c) only medium. The experiments were monitored by cell counts, VSV-specific immunofluorescence, and infectious VSV titers.

2. Results: The data shown in Figure 9 demonstrate that autogenous interferon produced by Ogun cells does protect SK-L1 cells against VSV. The protection of SK-L1 cells by virus-induced interferon was included for comparative purposes. It was evident that the SK-L1 cells exposed to autogenous interferon were as well protected for the first three days as the cells treated with virus-induced interferon. Infection of control cultures with VSV led to rapid death of the cells, a high incidence of cells stainable by fluorescent anti-VSV conjugate (50% on day one), and virus yields ranging from $10^{6.7}$ to $10^{7.2}$ TCD_{50} per ml. In contrast, the cells treated with autogenous interferon were largely protected for the first three days. This protection was evident by an only slightly reduced cellular growth rate as compared to that of the interferon control, virus-specific immunofluorescence was observed in less than 1.0% of the cells, and little infectious VSV was detectable. However, by day 4, the growth rate of the cells was more markedly reduced (by 35%) and on day 6 by 50%. This reduction in growth rate was paralleled by a rise in infectious virus titers but only a slight gradual increase in cells showing virus-specific immunofluorescence. The three parameters used to monitor the experiments showed that the cells treated with virus-induced interferon were significantly protected for the duration of the experiment.

The results given in Table XV illustrate the ability of SK-L1 or Ogun autogenous interferon to protect the cell cultures from which they were derived against challenge with VSV. The protection afforded in both instances was significant by all three parameters studied only for

FIGURE 9

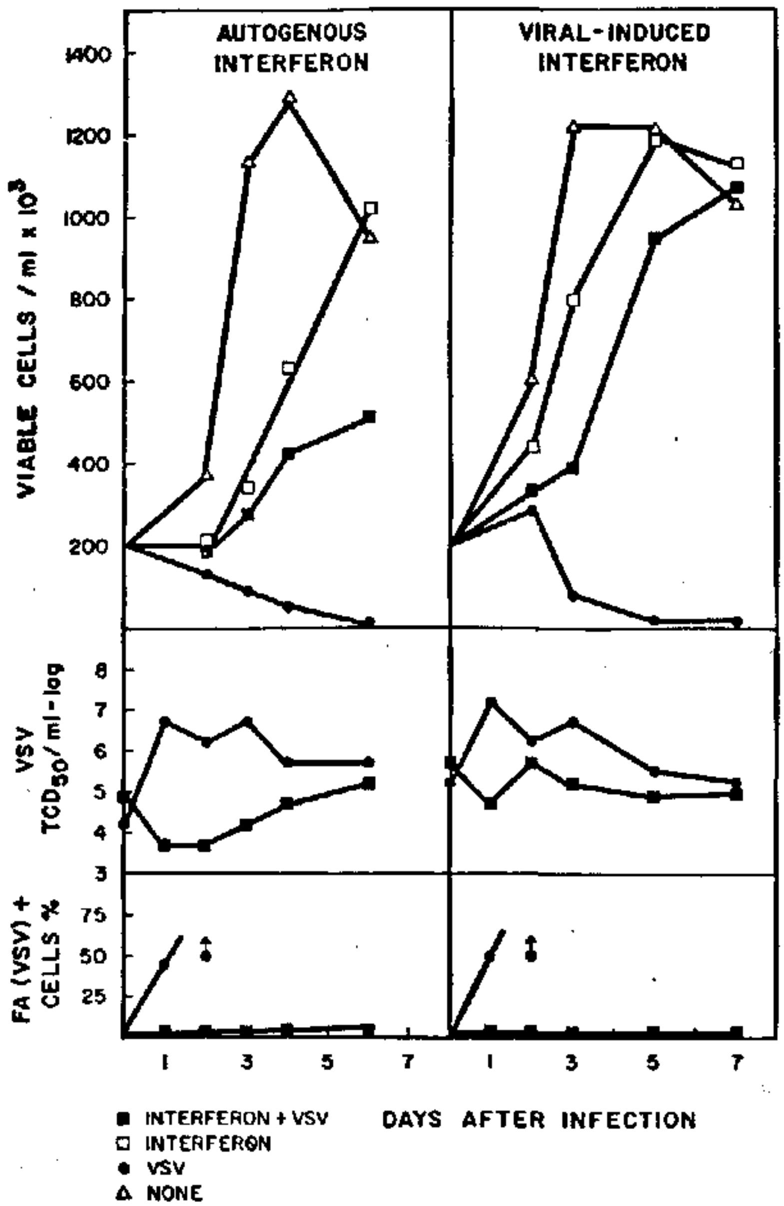

AUTOGENOUS INTERFERON
VIRAL-INDUCED INTERFERON
VIABLE CELLS / ml x 10^3
1400
1200
1000
800
600
400
200
VSV TCD50 / ml-log
8
7
6
5
4
3
FA (VSV) + CELLS %
75
50
25
1
3
5
7
DAYS AFTER INFECTION
INTERFERON + VSV
INTERFERON
VSV
NONE

TABLE XV

Capacity of autogenous interferon to protect cell **cultures** from which it was derived against VSV

Cells	Cells treated with	% viable cells[1]				VSV-TCD_{50}/ml log				FA(VSV) + cells %			
		1[2]	2	3	4	1	2	3	4	1	2	3	4
SK-L1 cells + SK-L1 IF[3]	VSV	100	52	7.5	4.0	5.7	6.2	6.2	4.7	1.42	14.8	18.4	29.8
SK-L1 cells	VSV	94	14	1.6	0.8	6.7	6.7	6.2	4.7	40.00	75.0	100.0	
Ogun cells + Ogun IF	VSV	100	60	32.0	4.2	5.2	6.7	6.2	5.2	0.91	17.8	10.0	10.0
Ogun cells	VSV	97	28	7.6	2.8	7.2	7.2	6.7	5.7	48.00	>50.0	75.0	100.0

(1) Percentage of viable cells as compared to interferon control
(2) Days after infection
(3) Interferon

the first two days. Thereafter cell death rapidly ensued, accompanied by an only slight increase in infectious virus titers. However, the percentages of cells showing VSV-specific immunofluorescence in the interferon treated cultures did not increase as sharply as might have been expected from the decrease in cell viability. The lack of increase in viral antigen could be related to a toxic property of VSV described by Cantell et al. (1962). These workers have shown that VSV at input multiplicities of 10 or greater seemingly overcame interference in NDV_{uv}-treated L cells in that the cells were destroyed as rapidly as in susceptible cultures, viral antigen formation was delayed, and no detectable amounts of infectious virus were produced.

These data indicate that autogenous interferon is capable of protecting the cells from which it is derived, and that the protection may be more transitory, than that attained by virus-induced interferon.

F. Discussion: The present study was undertaken to define more precisely the nature of the autogenous interferons produced by various Burkitt lymphoma and leukemia cell lines and to compare it with virus-induced interferons derived from the same type of cultures. The investigation included a study of the conditions for synthesis, the kinetics of synthesis, the properties, and the protective potencies of the two kinds of interferons.

The quantitative relationship between the yield of interferon and the number of cells per ml has been described by a number of investigators for the production of virus-induced interferon by human leucocytes (Gresser, 1961; Strander and Cantell, 1966; Wheelock, 1966). Initial experiments with Burkitt lymphoma cells showed that the yield of autogenous interferon was dependent on the cell concentration and independent of the type of medium and the serum concentration. By increasing the cell concentration maximal interferon titers were reached within 24 hours and the yields were further improved when the heavy cell suspensions were agitated during incubation. The amount of autogenous interferon produced was quite small, however, considering the number of cells utilized per culture. These low interferon yields suggest that possibly only a small percentage of cells in the population are involved in the production of autogenous interferon. Therefore, the amount of autogenous interferon produced by the VSV-susceptible cell lines was insufficient to protect them from VSV infection. Smith and Wagner (1967) also have reported that the yields of spontaneous interferon from uninfected macrophages were less than 1% of those obtained on viral induction of interferon synthesis.

In attempts to produce virus-induced interferon with NDV, NDV_{uv}, Sendai, Sindbis, influenza types A, A_2, B, vaccinia, St. Louis encephalitis and California encephalitis viruses it was surprising to find that only NDV and NDV_{uv} stimulated production of detectable amounts of interferon. Gresser (1961), Lee and Ozere (1965) and Strander and Cantell (1966) were able to obtain good yields of interferon from human leucocytes stimulated with Sendai virus. Vaccinia virus however failed to induce interferon synthesis in human leucocytes and influenza viruses were able to stimulate only minimal amounts (Strander and Cantell, 1966). Working with NDV as an inducer of interferon in human leucocytes Wheelock (1966) reported that an input multiplicity of 1 of viable NDV stimulated optimal yields of interferon. Strander and Cantell (1966) have shown that both viable and ultraviolet-inactivated NDV induced the synthesis of equivalent amounts of interferon at a given multiplicity. In the present experiments leukemia cells (SK-L1) required an input multiplicity of 10 of viable

NDV and NDV_{UV} to yield optimal amounts of interferon, while Raji cells required an input multiplicity of 50. The employment of an input multiplicity of 1 of viable NDV failed to yield optimal titers of interferon in any of the Burkitt lymphoma and leukemia cell lines tested.

Thus, the Burkitt lymphoma and leukemia cells seem to differ to some extent from human leucocytes in response to viral stimulation of interferon synthesis and, indeed, show some variations among themselves in this respect.

The quantities of virus-induced interferon obtained from certain of the Burkitt lymphoma and leukemia cell lines within 24 hours were 50-to 100-fold greater than autogenous interferon yields but in other instances the yields were of similar orders.

The kinetics of autogenous interferon production were found to be alike for both Burkitt lymphoma and leukemia cell lines. The early detection of autogenous interferon (within 1 hour of incubation) suggested that it might be preformed. The concept of preformed interferon had been introduced by Ho (1964a) to explain the rapid appearance of interferon in the circulation following intravenous inoculation of bacterial endotoxin into mice (Stinebring and Youngner, 1964) or into rabbits (Ho, 1964b). The presence of bacterial endotoxin in the culture medium of rabbit macrophages augmented the amount of spontaneous interferon produced (Smith and Wagner, 1967). However, this spontaneous interferon was not considered preformed since its production was blocked by actinomycin and puromycin. The treatment of SK-L1 and Raji cells with 100 ug of endotoxin (Lipopolysaccharide B, E. coli 0111:B4, Difco Laboratories, Detroit, Michigan) per ml failed to increase the production of autogenous interferon or induce interferon synthesis, respectively. Attempts to block autogenous interferon production in SK-L1 cells by puromycin dihydrochloride (Nutritional Biochemicals Corp., Cleveland, Ohio) gave less clear cut results than those designed to inhibit virus-induced interferon production. The presence of 50 μg per ml of puromycin in the medium at the time of addition of NDV_{uv} was sufficient to block synthesis of all virus-induced interferon. However, the addition of the same amount of puromycin to cells prepared for autogenous production reduced the amount of interferon obtained but did not block completely its synthesis. Pretreatment of these cells for one hour prior to preparation for autogenous production gave similar results. It would appear that the autogenous interferon produced by SK-L1 cells might be preformed but further studies are required to verify this point. It is quite conceivable that in some of these lines the in vitro cultivation of the cells triggers derepression of the usually repressed capacity of the cells to synthesize interferon.

The experiments performed to compare the time of appearance of virus-induced interferon in the various cell lines revealed a number of different patterns of interferon synthesis. SK-L1 and Ogun cells showed a very rapid synthesis of interferon with maximal titers at 8 hours, similar to the kinetics of virus-induced interferon production in rabbit macrophages (Smith and Wagner, 1967). Raji and 6410 cells showed a lag of at least 8 hours prior to the detection of interferon. A similar pattern has been reported for chick embryo cells stimulated by Semlikie Forest virus (Friedman, 1966). The third pattern examplified by Jijoye and EB-1 cultures was intermediary in that it showed a lag of 2 to 3 hours with peak yields being reached between 8 and 16 hours. These kinetics seem to be similar to those observed in virus-induced interferon production by human leucocytes (Wheelock, 1966).

The three patterns of synthesis may be due in part to the heterogeneity of the cell types, genetic factors, physiological conditions, responses of the various cells to the inducing viruses, activation of different mechanisms of synthesis and release, or to the presence of autogenous interferon. Reduction in the lag period of interferon synthesis as well as increases in interferon titers have been shown by various workers when cells were pretreated with small amounts of interferon (Lockart, 1963; Friedman, 1966; Levy et al., 1966). The presence of autogenous interferon in Burkitt lymphoma and leukemia cell lines appears to have enhanced the time of appearance and rate at which virus-induced interferon is produced, but not necessarily the yield of interferon.

Attempts to distinguish the autogenous from virus-induced interferons by a variety of chemical and physical treatments revealed no significant differences in their behavior. However the molecular weights of these interferons may vary since it has been demonstrated that non-virus induced and virus-induced interferons were of different molecular weights (Hallum et al., 1965; Ho, 1964b; Wheelock, 1965). Youngner et al. (1966) have also reported that the interferons found in mouse serum differed in time of appearance and molecular weights depending on the stimulus employed and the time of bleeding. Such studies are now planned for the autogenous and virus-induced interferons of Burkitt and leukemia cell lines.

Ogun and SK-L1 cells which produced autogenous interferon and yet were fully susceptible to VSV provided a paradox which remained to be resolved. It was found that these cells could readily be protected against VSV by addition of extraneous, virus-induced interferon. It was likely, therefore, that too few of the cells produced autogenous interferon and the amount was insufficient to protect a substantial proportion of the non-producing cells. This interpretation is supported by the observation that concentrated autogenous interferons were capable of protecting cultures of the cell lines from which they were derived. However, protection was weaker than that induced by comparable units of virus-induced interferon, suggesting that autogenous interferon might be less efficient in establishing resistance.

The results presented have shown that autogenous and virus-induced interferon, obtained in part from the same cell line, were indistinguishable according to the criteria used. Further studies on differentiating the two interferons will have to include metabolic inhibitors and molecular weight determinations.

III. The Effect of Mumps Virus on Burkitt Lymphoma Cell Lines.

It has not been possible in the past to increase the yield of the herpes-type virus in cultures of Burkitt tumor cells or to transmit it to other host systems for growth in quantity. These failures could be due largely to the fact that the virus particles were largely defective. Because of this situation it is not known yet whether this agent is merely a passenger in these cultures or whether it is etiologically related to Burkitt's lymphoma and leukemia, for that matter, since similar virus particles have been found also in cell lines derived from leukemic bone marrows and peripheral blood. Efforts are being made in this and other laboratories to improve the yield of virus by variations in cultural procedures or by providing helper effects, in their broadest sense, by superinfection with other viruses. As to the second approach, previous studies (Section I) have revealed that many of the cell lines exhibited a remarkable resistance to viral infections which could be augmented by production of autogenous interferon in some of the cultures. Several reports have indicated that members of the paramyxogroup of viruses may lower the resistance of cell populations (Kumagai, 1958; Hermodsson, 1963; Valle and Cantell, 1965; Frothingham, 1963; Kato et al., 1965; Maeno et al., 1966) and that this effect may be due to reduced interferon synthesis (Hermodsson, 1963; Maeno et al., 1966) or inhibition of the action of interferon (Hermodsson, 1964; Cantell and Valle, 1965; Frothingham, 1965). Several of the lymphoma cell lines were therefore exposed to mumps virus in an attempt to determine its effect upon the resistance of the cells to the herpes-type virus carried by them.

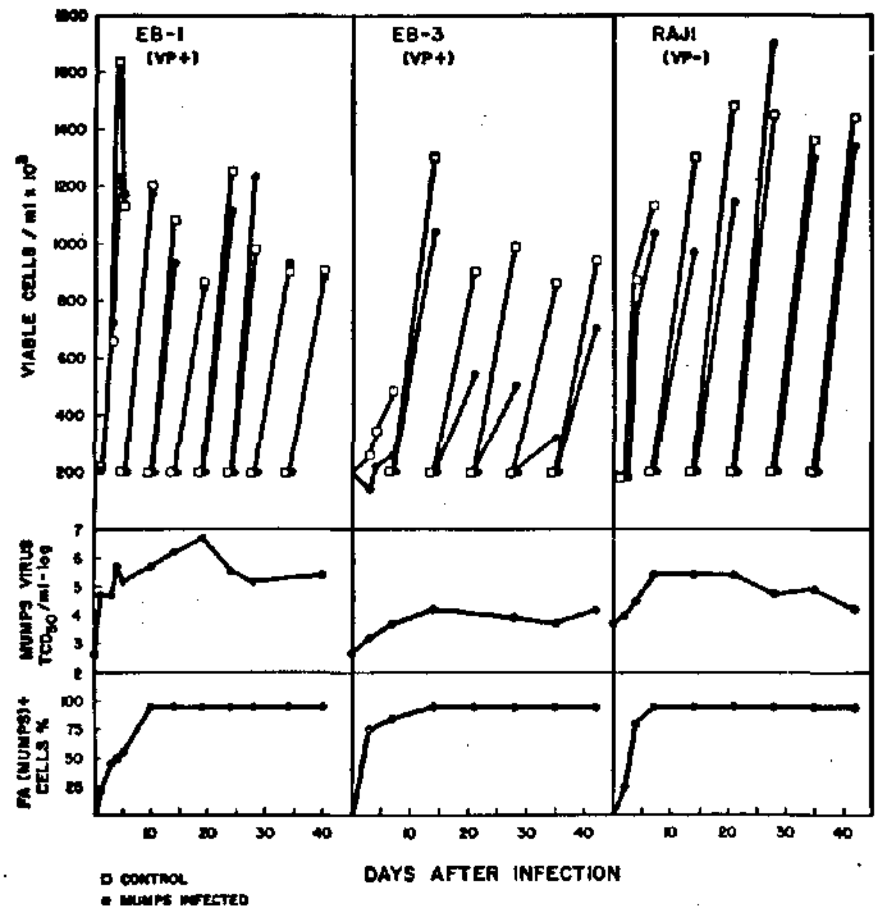
A. <u>Infection of cultured Burkitt cells with mumps virus</u>: The cell lines selected for these studies were (1) the EB-1 line which contained herpes-type virus particles and produced autogenous interferon; (2) the EB-3 line which carried the unidentified agent but did not produce detectable amounts of autogenous interferon; and (3) the Raji line which neither contained virus particles nor did it produce autogenous interferon. The course of mumps virus infection in these cell lines was studied first.

1. <u>Procedure</u>: 5×10^6 of the respective cells were sedimented by centrifugation at 1000 rpm for 10 minutes. The medium was decanted and the pellet resuspended in 2.0 ml of mumps virus diluted to give an input multiplicity of about 5 based on HAU per ml and assuming 1 HAU to be equivalent to 10^6 TCD_{50}. The cell-virus mixture was incubated with intermittent agitation for 2 1/2 hours at 37 C and then centrifuged. The pellet was resuspended in growth medium to yield a cell concentration of 2×10^5 per ml. The cultures were sampled immediately in order to determine the number of viable cells and background virus and were then incubated at 37 C. Uninfected control cultures were handled and maintained in parallel. At varying time intervals after infection the following procedures were carried out: (a) total and viable cell counts; (b) preparation of smears for staining with fluorescein-conjugated mumps hyperimmune human gamma globulin, and determination of the percentage of cells containing mumps antigen(s); and (c) collection of samples of cell suspension in triplicate for storage in ampules at -70 C for future assay of infectious mumps virus. In experiments extending over more than seven days, the cell density of the cultures was reduced at weekly intervals to 2×10^5 per ml by dilution in appropriate amounts of fresh growth medium.

2. __Results:__ Figure 10 compares the effect of mumps virus on the three Burkitt cell lines. The EB-3 line was most markedly affected by the infection. The cellular growth rates were reduced, at various intervals by 18 to 63% as compared to uninfected control cultures with an average reduction of 48%. The EB-1 and Raji cell lines showed an average reduction in cell multiplication of 20% and less than 10% respectively.

The titers of mumps virus in the EB-3 cell line rose within 7 days to about 10^4 TCD_{50} per ml and thereafter remained at this level for the period of observation. In the Raji cell line the titers reached an approximately 10-fold higher level and in the EB-1 line the levels of mumps virus rose gradually over a 20 day period to a peak of $10^{6.7}$ TCD_{50} per ml and then declined to values slightly above 10^5 TCD_{50} for the remainder of the observation period. The actual amounts of virus produced were probably about 100-fold higher since comparative titrations of the Ricki strain of mumps virus in HEK cells and in chick embryo amnion revealed generally differences of about 2 $\log_{10}$ units in favor of the latter host system. Even with a corresponding correction the titers were still surprisingly low in view of the fact that within 5 to 10 days practially all cells contained mumps antigen as evident from mumps-specific immunofluorescence. It is obvious from the cellular growth rates that infected cells retained their capacity to divide. These results resemble therefore the persistent mumps infection of Chang's human conjunctiva cell line described by Walker and Hinze (1962a). The few non-staining cells observed possibly had been dead for some time and any viral antigen present might have been thermally denatured. Only a few of the cells

FIGURE 10


EB-1
(VP+)
EB-3
(VP+)
RAJI
(VP-)
VIABLE CELLS / ml x 10³
MUMPS VIRUS TCD50/ml-log
FA (MUMPS)+ CELLS %
DAYS AFTER INFECTION
□ CONTROL
● MUMPS INFECTED

contained enough mumps antigen to render the whole cell intensely fluorescent. These were mainly seen during the early incubation period. The great majority of cells contained well delinated aggregates of antigen of varying size (Figure 11) often amounting to minute granules. This was indicative of an abortive infection.

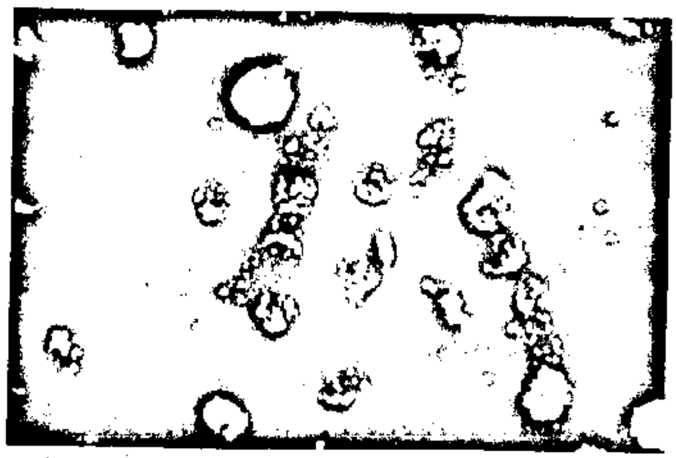
Some of the infected cultures have been maintained for more than 3 months without apparent change in the quality of immunofluorescent staining and in the virus titers. A slight degree of periodicity in the cellular growth rates was evident in some of the infected cultures suggested also by the EB-3 data in Figure 10, in that periods of more marked reduction were followed by periods of better growth.

In their studies with mumps-infected human conjunctiva cells Walker and Hinze (1962b) were able to increase infectious virus titers in the medium and the number of infected cells showing hemadsorption of chicken erythrocytes, by reducing the serum concentration from 10 to less than 3% at the onset of infection. These investigators also obtained enhanced virus titers at later stages of infection if the serum concentration was lowered (Walker et al., 1966). Attempts to alter the course of the mumps virus infection in EB-1 cultures at the time of exposure or at a later stage by reducing the serum concentration to 2% were unsuccessful. Under these conditions no changes were seen in the type of mumps-specific immunofluorescence nor was an enhancement of infectious mumps virus titers observed when compared to the response of cultures maintained on 10% serum.

B. Effect of mumps virus on the indigenous herpes-type virus: Since mumps and other paramyxoviruses had been shown to enhance replication of a number of viruses by inhibition of the action of interferon (Hermodsson, 1964; Cantell and Valle, 1965; Frothingham, 1965), EB-1 and EB-3

FIGURE 11

Mumps-specific immunofluorescence in EB-1 cells infected 15 days previously. Magnification X 1,250.



cells infected with mumps virus were monitored, at various times after infection, by immunofluorescence specific for the herpes-type virus, and inoculations of various types of tissue cultures and newborn and weanling animals in an effort to detect enhancement of the indigenous herpes-like virus.

1. Procedures: EB-1 and EB-3 cultures were infected with mumps virus as described in section A. After 2, 5, 7, and 14 days of incubation infected and control cell suspensions were harvested. Samples were removed for immunofluorescence tests and ampules containing cell suspensions were shell frozen and stored at -70 C for tissue culture and animal inoculations. An EB-3 mumps carrier culture initiated 50 days previously was also used for tissue culture inoculation.

a. Immunofluorescence: Cell smears derived at intervals from mumps infected cultures and controls were stained not only for mumps antigen but also for the indigenous virus. A fluorescein-labeled human mumps hyperimmune globulin (Henle and Henle, 1966a) was used after exhaustive adsorption with mumps virus. This preparation failed to detect the characteristic mumps antigen aggregates but retained its capacity to stain the appropriate proportion of EB-1 and EB-3 cells harboring herpes-type virus (1-2% and 3-5%, respectively).

b. Inoculation of tissue cultures: The various samples of cells were thawed and exposed to sonication for 3 minutes. The sonicated cell suspensions were mixed with equal amounts of a 1:5 dilution of equine antimumps serum and incubated one hour at room temperature. Test tube cultures, with and without floating coverslips, of HEK, WI-38, primary rabbit kidney, fat head minnow, and turtle heart were inoculated with 0.2 ml per tube of given mixtures. The cultures were observed for cytopathic effects and development of viral interference as tested by challenge with 100-1000 TCD_{50} of VSV. At various intervals coverslips were harvested and processed for immunofluorescence.

c. Animal inoculations: The cell suspensions were sonicated and treated with equine antimumps serum as indicated above. Litters of 1 and 6 day old hamsters and mice were inoculated intraperitoneally or intracerebrally with 0.03 ml per animal. The animals were checked initially daily for illness or death and later at longer intervals for development of tumors.

2. Results: Cell smears prepared directly from the infected Burkitt cell lines failed to show at anytime in the course of the experiments any significant increases in the proportions of EB-1 or EB-3 cells harboring herpes-type particles.

None of the inoculated cell cultures showed cytopathic effects (CPE) referable to the herpes-type virus. However, in a few instances cytopathic effects were noted which were found to be due to unneutralized mumps virus. Cultures without CPE were maintained by refeeding and reseeding for as long as 40 days and showed no evidence of interference with VSV when tested at various intervals. The coverslip cultures failed to reveal any immunofluorescence specific for the herpes-type virus.

None of the mice inoculated at birth or later developed any signs of illness. About 12% of the hamsters infected at the newborn stage became ill or were found dead after incubation periods of 10 to 17 days. Two of the preparations were shown to contain unneutralized mumps virus in the tissue culture studies. Passages of brain or spleen homogenates from these animals into newborn animals and cell cultures failed to produce any illness or to show any CPE. Surviving animals of the primary and passage series were kept for 18 months and observed for evidence of tumor formation. None became detectable.

C. Demonstration of lowered resistance in mumps carrier cultures: The above experiments failed to reveal an effect of mumps virus infection on the indigenous virus. It was essential therefore to demonstrate that mumps virus indeed was capable of lowering the resistance of Burkitt cells to other viruses.

Preliminary experiments had shown that cells from an EB-1 line which had been infected 15 days previously with mumps virus ($EB\text{-}1_{mumps}$) were more susceptible to VSV than mumps virus-free control cultures. This was evident from (a) a significantly greater reduction in cellular growth rate during the first week after challenge with VSV (about 75% versus 10% in the control); (b) larger number of cells revealing VSV-specific immunofluorescence (40% as compared to 4%); and (c) greater than 10 times higher yields of infectious VSV in 24 hours ($10^{7.2}$ versus $10^{5.7}$ TCD_{50} per ml). Following refeeding there was, however, a decided improvement in cellular growth, a decline in VSV-specific immunofluorescence (to 12-25%) and a decrease in infectious VSV titers. The cultures survived and carried now 3 viruses, the herpes-type, mumps, and VSV viruses. Throughout the observation period the staining for mumps antigen remained constant, showing >95% of cells to be infected, and the mumps virus titers fluctuated around 10^5 TCD_{50} per ml. Staining of the cells for the indigenous herpes-type virus revealed the usual 0.5 to 2% positive cells.

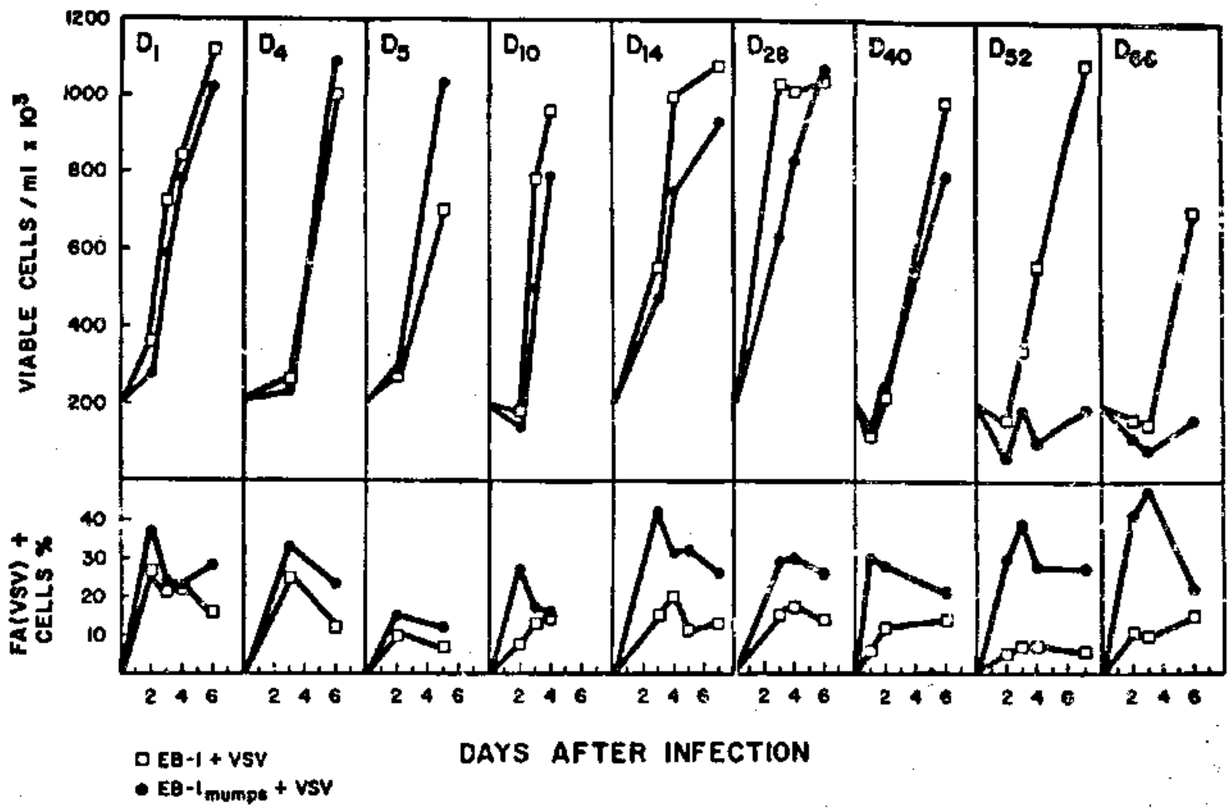
These preliminary observations have shown that a mumps infection was indeed able to lower to some extent the resistance of EB-1 cells to infection with VSV. It was conceivable that during a prolonged mumps viral carrier state the susceptibility of the cells might be increased further resulting possibly in the ultimate destruction of the Burkitt cells by VSV.

1. <u>Acquisition of enhanced susceptibility to VSV with prolongation of the mumps viral carrier state:</u>

a. <u>Procedure:</u> EB-1 cultures were infected with mumps virus and at various intervals cells from these, as well as uninfected populations, were centrifuged and the pellets resuspended in 1.0 ml of VSV diluted to achieve an input multiplicity of 2. After an adsorption period of 1 hour, the cells were centrifuged and resuspended in sufficient growth medium to yield 2×10^5 per ml. The experiments were monitored by cell counts, immunofluorescence, and infectivity titers.

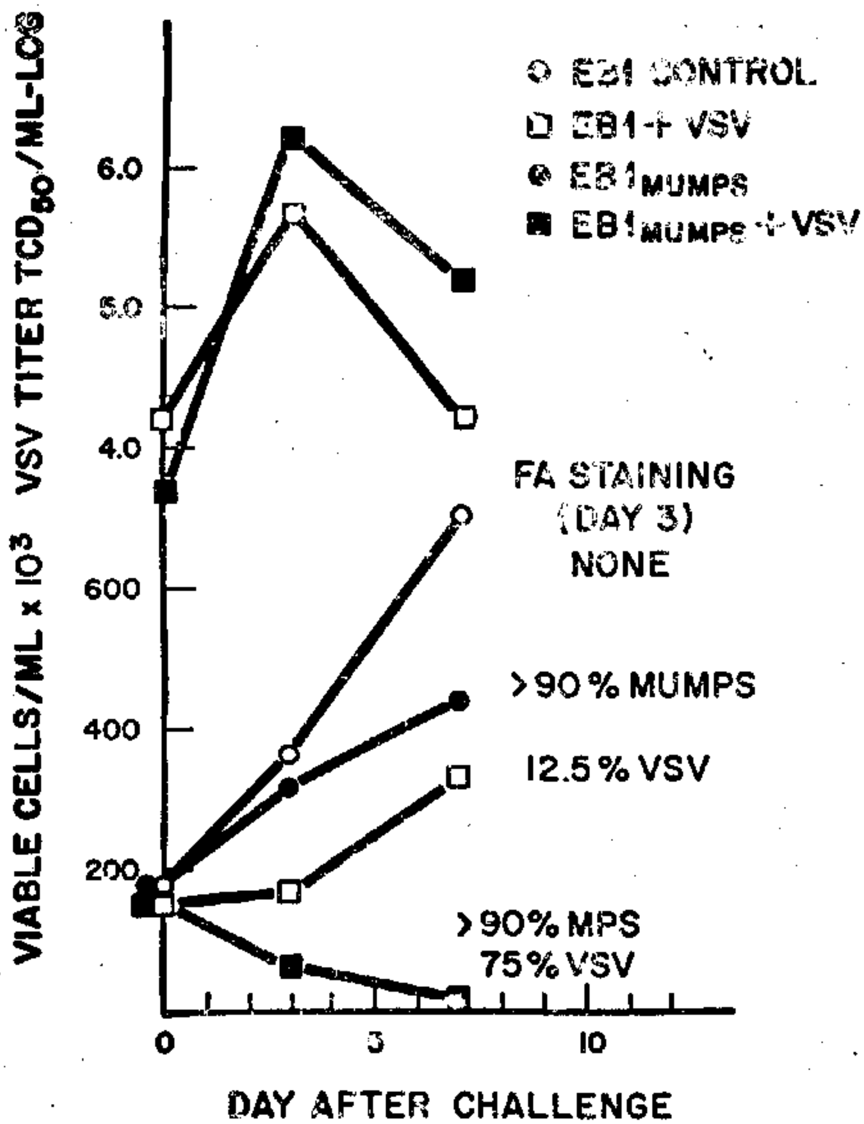
b. <u>Results:</u> Figure 12 demonstrates the gradual acquisition of an enhanced susceptibility of $EB\text{-}1_{mumps}$ cultures to superinfection with VSV. The enhancement in terms of reduction in cell growth was not dramatically evident before 52 days following exposure to virus. At 52 and 66 days the effect on cell growth was rapid and pronounced. However, low levels of enhancement were evident from VSV-specific immunofluorescence already on the first day after infection with mumps virus. The difference in VSV antigen-containing cells between the experimental and control groups gradually increased so that by day 14 the enhancement was already seen. In each VSV challenge, the enhancement was always most evident within the first 3 days after superinfection, since thereafter the infection in the controls declined toward the levels known to persist indefinitely as shown in Section I. It was unfortunate that this particular mumps carrier culture was lost due to bacterial contamination. Another $EB\text{-}1_{mumps}$ carrier culture had been maintained, however, for a similar length of time and as can be seen in Figure 13, this culture was destroyed within 7 days after infection with VSV.

FIGURE 12



VIABLE CELLS / ml x 10³
FA(VSV) + CELLS %
D1
D4
D5
D10
D14
D28
D40
D52
D66
DAYS AFTER INFECTION
□ EB-I + VSV
● EB-I mumps + VSV

FIGURE 13


EB1 CONTROL
EB1 + VSV
EB1 MUMPS
EB1 MUMPS + VSV
VSV TITER TCD50/ML-LOG
VIABLE CELLS/ML x 10³
6.0
5.0
4.0
600
400
200
FA STAINING
(DAY 3)
NONE
>90 % MUMPS
12.5 % VSV
>90% MPS
75% VSV
0
5
10
DAY AFTER CHALLENGE

Similar but less extensive experiments were carried out with EB-3 and Raji cells. Three weeks after mumps infection EB-3 cultures on VSV challenge gave results comparable to those obtained with EB-1$_{\text{mumps}}$ carrier cultures at 15 days. With Raji cells, which do not harbor the herpes-like virus, a corresponding experiment failed to reveal significant differences in susceptibility to VSV between the control and mumps infected cells. The mumps infection was of three weeks duration when the cells were challenged. If anything the growth rate of the Raji$_{\text{mumps}}$ population was somewhat less affected by VSV than the Raji control. Virus titers and the percentage of cells showing VSV-specific immunofluorescence were throughout of a similar order and both cultures were maintained for three months as VSV and VSV plus mumps carriers, respectively. Another superinfection of Raji$_{\text{mumps}}$ cultures with VSV 90 days after infection with mumps also failed to reveal significant differences in susceptibility to VSV between the control and mumps carrier cells.

D. <u>The effect of mumps virus infection on the production and action of interferon in Burkitt lymphoma cells</u>: Frothingham (1965) showed that mumps infection inhibited the protective action of interferon in chick embryo fibroblast cultures as assayed by challenge with Sindbis virus, but it did not inhibit the production of interferon in this system. Other investigators have shown that different paramyxoviruses inhibited the production of interferon (Maeno, et al., 1966), the action of interferon (Cantell and Valle, 1965), or both (Hermodsson, 1963, 1964). In order to determine whether a mumps viral carrier state in Burkitt cell cultures affects either the production of interferon by these cells or their protection by an extraneous interferon the following experiments were designed. $Ogun_{mumps}$ and Ogun control as well as $Raji_{mumps}$ and Raji control cultures were used for experiments on interferon production, and $EB\text{-}1_{mumps}$ and EB-1 control cultures were employed to determine whether mumps-infected cells can be protected by extraneous human interferon against challenge with VSV.

1. Procedures:

a. Production of interferon: In order to determine the effect of a mumps viral carrier state on autogenous interferon production various mumps-infected and control cells were centrifuged at 1000 rpm for 10 minutes. The cells were resuspended in maintenance medium to yield 1×10^7 cells per ml and incubated on a rotary shaker.

To determine the effect of a mumps carrier state on virus-induced interferon production $Raji_{mumps}$ and control Raji cells were counted and 1×10^8 cells of each were centrifuged and resuspended in 5.0 ml of NDV_{uv} at an input multiplicity of about 50. After an adsorption period of one hour at 37 C with continuous agitation, 5.0 ml of maintenance medium was added and the cultures were incubated on a rotary shaker for 24 hours. The media were harvested and processed for interferon assay.

b. Protection by interferon: $EB\text{-}1_{mumps}$ cultures, infected 66 days previously, and control cultures were exposed to 170 units per ml of interferon produced by SK-L1 cells on stimulation with NDV_{uv}. The cells were centrifuged, resuspended in the interferon preparation, incubated for 24 hours and then challenged with VSV at an input multiplicity of 2. After an adsorption period of 2 hours the volume of the cultures was adjusted to yield 2×10^5 cells per ml. Control cultures carried in parallel were treated with (a) only VSV; (b) only interferon; and (c) only medium. The experiment was monitored by cell counts, VSV-specific immunofluorescence, and infectious VSV titers.

2. Results: Production of interferon: The data presented in Table XVI show that infection of EB-1, Ogun, and SK-L1 cells with mumps virus resulted in no inhibition of synthesis of autogenous interferon. Both infected and uninfected cells, carried in parallel, produced equivalent amounts (16 to 32 units per ml) of autogenous interferon. In contrast, Raji cells previously infected with mumps virus failed to synthesize any detectable amounts of interferon when exposed to NDV_{uv}, whereas Raji control cultures, stimulated simultaneously, produced 256 to 512 units of interferon per ml. The lack of any detectable interferon synthesis by $Raji_{mumps}$ cells might be attributable to destruction by mumps virus of cell receptors for NDV since it has been demonstrated (Burnet, 1955) that mumps virus will partially remove receptors from red blood cells needed for agglutination of these cells by NDV. Fluorescent antibody studies with FITC-conjugated antibodies to NDV showed, however, that $\geq$ 13% of the $Raji_{mumps}$ cells became infected with NDV within 16 hours. At the same time 35% of the control Raji cells contained NDV antigen. This difference in the percentages of NDV-infected cells hardly can account for the difference in interferon production. To clarify this point it would be essential to use other viruses as inducers of interferon synthesis. This has not been possible since earlier studies (Section II) had shown that three strains of influenza viruses, vaccinia virus, and two arbor viruses failed to induce any detectable interferon synthesis in Raji cells. Studies with other cell lines (EB-1 and

TABLE XVI

Effect of mumps-virus infection on the production of autogenous and virus-induced interferon

Cell line	Type of interferon	Interferon units per ml	
		Control	Mumps-infected
EB-1	Autogenous	32	32
EB-1	"	16	16
Ogun	"	24	24
SK-L1	"	32	32
Raji	Virus-induced	512*	<4*
Raji	"	256	<4

*The number of control and mumps-infected cells showing NDV-specific immunofluorescence 16 hours after stimulation were 35 and 13%, respectively.

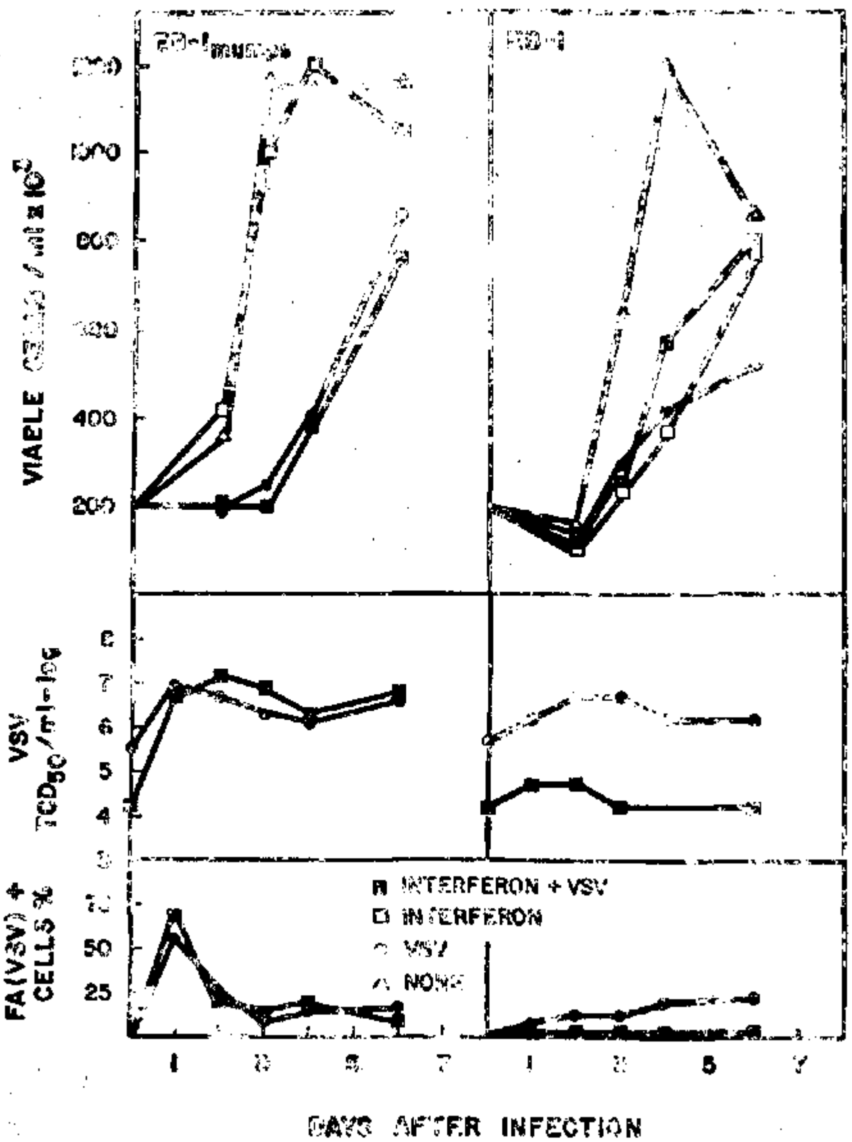
Ogun) have shown that infection of 10% of the cells with NDV was sufficient to induce production of at least small amounts of interferon. Thus the $EB\text{-}1_{mumps}$ cells should have been able to produce some interferon when induced by NDV_{uv}.

Protection by extraneous interferon: The results given in Figure 14 clearly show that mumps-infected EB-1 cells could not be protected by exogenous interferon against challenge with VSV, whereas EB-1 cells free of mumps virus were protected. In the $EB\text{-}1_{mumps}$ culture cellular growth rates, VSV-specific immunofluorescence, and VSV-infectivity titers were parallel in both interferon treated and control cultures. In contrast, EB-1 cells (free of mumps virus) treated with interferon showed the usual reduction in these parameters as compared to untreated control.

These experiments have shown that a mumps carrier state in Burkitt cultures failed to influence autogenous interferon production, possibly reduced the capacity to synthesize virus-induced interferon and prevented the protective action of interferon.

E. Discussion: The results of mumps infection in the Burkitt lymphoma cell lines tested resembled the persistent mumps infection of Chang's conjunctiva cell line described by Walker and Hinze (1962a). Even at a high multiplicity of infection there was relatively little evidence of cell loss and the cultures showed merely a reduction in growth rates which were most marked with the EB-2 line and barely perceptible with Raji cells. Practically all cells were found to contain mumps antigen but this was mostly aggregated into well-delineated masses of variable sizes indicative of an abortive infection. The cells evidently were not greatly disturbed and continued to divide yielding infected daughter cells. This type of carrier state has been termed "regulated infection" (Walker, 1964) or endosymbiosis (Fernandes et al., 1964), and has been observed in addition

FIGURE 1b


RD-1
VIABLE
400
200
VSV
7
6
5
4
50
25
INTERFERON + VSV
INTERFERON
VSV
NONE
DAYS AFTER INFECTION

to mumps virus with parainfluenza type 1 (Ishida et al., 1964), measles virus ([illegible], 1966), and rabies virus (Fernandes et al., 1964). The exact mechanism(s) which permit the survival of both cells and virus are presently unknown.

Infection of cell cultures by paramyxoviruses; e.g. mumps, parainfluenza or Newcastle disease viruses, has been shown to lower the resistance of various cell cultures to a number of other viruses (Kumagai, 1959; Frothingham, 1963; Hermodsson, 1963; Kato et al., 1965; Valle and Cantell, 1965; Maeno et al., 1966). The mumps-infected EB-1 cells showed a greater susceptibility to VSV than mumps-free control cultures. Using EB-3 cells the enhancing effect was less marked but evident, while no enhancement could be detected in Raji cells. Henle and Henle (unpublished data) have noted that mumps infected EB-1 cells also showed a greater susceptibility to herpes simplex virus.

The parainfluenza group of viruses may act as helper viruses by suppressing the production and/or the action of interferon (Cantell, to be published). The data reported here have shown that mumps infection of EB-1, SK-L1, and Ogun cells did not affect the production of autogenous interferon. Mumps viral carrier cultures of Raji cells failed to produce virus-induced interferon, but the possibility has not been entirely ruled out that the failure might not be due to receptor destruction. For a final answer other viral inducers must be found which are effective in these cells. None of the 8 other viruses often employed for interferon induction were effective in Raji cultures. The mumps carrier state of EB-1 cell cultures clearly prevented the protective action of an exogenous interferon. Similar results were reported by Frothingham (1965) who showed that inhibition of interferon action resulted in chick embryo fibroblasts after mumps virus infection without noticeable inhibition of interferon synthesis.

In spite of the increased susceptibility to VSV and HSV of [illegible], HA-1 and HE-2 lines after establishment of a mumps viral carrier state, there was no obvious change in the herpes-type viral infection of these cultures. The percentages of cells harboring this agent on the basis of immunofluorescence remained stationary. Renewed attempts to transmit the herpes-type virus infection with materials collected from mumps carrier cultures at various times after their initiation and mixed with anti-mumps serum prior to inoculation into various types of cell cultures and newborn or weanling mice and hamsters failed to provide any evidence of success.

SUMMARY AND CONCLUSIONS

The data presented have shown that continuous cell cultures derived from similar specimens and maintained under identical conditions may differ considerably in their activities. The various Burkitt tumor cell lines, composed of lymphoblasts which may differ to some extent in the degree of maturity of the cells, are nevertheless so closely similar that it would be difficult indeed to identify a given line on the basis of morphology alone. The cell lines derived from leukemic patients, regardless of the type of leukemia, or from healthy donors also are difficult to differentiate on morphological grounds from each other as well as from the Burkitt tumor cell lines. This raises the question as to the actual nature of the cells cultured from the various specimens which deserves intensive study.

Some of the cell lines under study were known to harbor herpes-type virus particle and others not. Some were found to be resistant to VSV and/or HSV and others were not. Finally some revealed the presence of an interferon and others not. These properties were not necessarily interrelated. While in some instances the herpes-type viral carrier state was apparently accompanied by resistance and interferon production, other infected cultures were susceptible to the extraneous viruses and failed to yield interferon. Cultures free of herpes-type virus were, mostly, highly susceptible to infection by the 2 challenge viruses, yet a few were found to be resistant. Some of the susceptible cell lines devoid of herpes-type virus produced an apparently autogenous interferon, that is, without apparent stimulation. Thus, no correlation could be established among the above properties. All but one of the cell lines were capable

Some of the cultures which produced autogenous interferon remained nevertheless highly susceptible to VSV. This suggested that only few of the cells were interferon producers and the amount of inhibitor synthesized was too small to protect a significant proportion of the cells. This interpretation is supported by the observation that concentrated autogenous interferon was capable of protecting cultures of the cell line from which it was derived. Some indication was found that unit for unit virus-induced interferon is slightly more protective than autogenous interferon, at least with respect to the duration of the resistance induced.

Further studies on differentiating the autogenous and virus-induced interferons, should evaluate the effects of metabolic inhibitors on the synthesis of these two interferons and compare their molecular weights. Attempts were made to study inhibition of protein synthesis but these were hampered, not unexpectedly, by the asynchrony of autogenous interferon synthesis and the toxicity of the compound which did not permit prolonged treatment of the cultures. Since it has been noted that "preformed" interferons possess higher molecular weights than virus-induced ones, it would be of interest to determine whether autogenous interferon conforms to the former. If so, it would suggest that one source of preformed interferon in animals might be blast cells of the hematopoietic system.

It has been possible to reduce the resistance of Burkitt tumor cell lines to VSV by superinfection with mumps virus. All cells became infected with this agent and the cultures survived and continued to grow as dual carriers of mumps and herpes-type viruses. In the course of the mumps infection the cultures showed gradually increasing susceptibility to VSV. During the first 2 months after exposure to mumps the cultures survived the VSV infection, but later superinfection with VSV led to their destruction. The mumps infection did not influence autogenous interferon synthesis, but it apparently reduced virus-induced interferon production and prevented the protective action of added virus-induced interferon. In spite of the evidence for reduced resistance to VSV and HSV the mumps carrier state did not affect the extent of the infection by the herpes-type virus. The percentage of herpes-type virus-infected cells remained constant on the basis of specific immunofluorescence and renewed attempts to transmit the agent, after neutralization of the mumps virus, to various cell cultures, newborn or weanling mice and hamsters were again unsuccessful. Improved replication of the herpes-type virus by variations in cultural conditions or with the aid of other potential "helper viruses" remains a foremost goal so that the herpes-type virus may be ultimately transmitted to other types of cell cultures and produced in bulk for study of its properties, especially its oncogenic potential.