IDENTIFICATION AND CHARACTERIZATION OF A HERPES VIRUS ISOLATED FROM BURKITT LYMPHOMA CELLS IN CULTURE

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ABSTRACT

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By Charles Alan Bowles

Some biological, physical and serological properties of a newly isolated virus were determined. The virus, designated MDH-1, was isolated from Burkitt Cells (line P3-J) in culture ten days after superinfection with Moloney sarcoma virus (MSV). Serologic studies indicated the virus to be dissimilar to MSV and to human and canine herpes virus but had a strong antigenic relationship to infectious bovine rhinotracheitis virus (IBR). Comparison of the ultrastructure of MDH-1 and IBR viruses by electron microscopy revealed many similarities in structure and developmental pattern. Host range studies indicated a difference in tissue culture cell susceptibility of human kidney, canine embryo and Earle's L-929 mouse fibroblasts to the two viruses. The serologic results, electron microscopic and host range data suggested that MDH-1 and IBR viruses probably represent two strains of the same virus.

Examinations of MDH-1, IBR and particles from the P3-J cells after centrifugation in a potassium citrate gradient revealed these viruses to have a density of 1.20. Concentrated herpes-like particles, removed from the P3-J cells, fixed complement in the presence of specific anti-MDH-1 serum. Sera from patients with lymphoma produced a positive, indirect, immunofluorescent reaction with bovine cells infected with

MDH-1 virus. Sera from patients with infectious mononucleosis also produced a positive fluorescent reaction. The serologic results, density, size and appearance as viewed by the electron microscope suggest that this agent was similar to the herpes-like virus previously reported by others in Burkitt cell cultures. The origin of MDH-1 and its possible relation to the cultured Burkitt lymphoma cells was discussed.

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By

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INTRODUCTION

A number of continuous cell cultures have been derived from tumors of individuals with Burkitt's lymphoma. Many authors have suggested a viral etiology for this tumor based on epidemiological findings and the demonstration by electron microscopy of particles morphologically similar to the herpes group of viruses in the cultured Burkitt lymphoma cells (Epstein et al., 1966; Stewart et al., 1965; Toshima et al., 1967; and others). Propagation of these herpes-like particles as productive viruses in cells other than the cultured Burkitt cells has been unsuccessful.

Efforts have been made to establish a serologic relationship between the herpes particles in cultured Burkitt lymphoma cells and other viruses and to other disease syndromes. A serologic reaction has been demonstrated between these agents and antibodies in the sera of patients with infectious mononucleosis but not to other known viruses. Attempts at determining some of the physical characteristics of these agents have been limited by the fact that the herpes-like viruses were not infectious by the techniques employed. Recovery of an infectious agent from the cultured Burkitt cells would provide a means of establishing the physical and biological characteristics, as well as the oncogenic capacity.

The present studies were concerned with the recovery of an infectious herpes virus from the cultured Burkitt lymphoma cells (P3-J line) and the identification and characterization of that agent by serological,

biological and physical means. Attempts to recover the herpes-like virus were based on the assumption that interaction with another virus was required for the enhancement of the noninfectious virus.

These investigations are presented in part as reprints of the publications appearing in Lancet and in the Journal of the National Cancer Institute and in part as a manuscript submitted for publication to the Journal of the National Cancer Institute.

LITERATURE REVIEW

Introduction

The demonstration by Ellerman and Bang (1903) that the erythromyeloblastic form of leukemia in chickens was transmitted by a cell free filtrate led investigators to search for viruses as causative agents of cancer in other animal species. The importance of this work was reemphasized by Gross (1951) with the recovery of a virus which was responsible for leukemia in mice.

Attempts to isolate a virus as the etiology of human malignancies has been under intensive investigation, but the isolation of such an agent has not been accomplished despite the frequent observation by electron microscopy of C-type virus-like particles, a specific morphological type of virus described by Bernhard and Guérin (1958), in malignant tissues from patients with leukemia and lymphoma (Dmochowski et al., 1959; Braunstiener et al., 1960; Almeida et al., 1963; Ames et al., 1966). The findings of herpes-like particles in cultured cells derived from tumors suggests another possible virus as the etiology of human malignancy (Epstein et al., 1964; Toshima et al., 1967).

One criterion, used to demonstrate the causal relationship of a virus to cancer, is the formation of malignant tumors in animals when inoculated with a suspect virus. Since this technique is not practical in humans, indirect procedures are necessary to relate a suspect virus to a malignant disease. These procedures may establish serological relationships between the virus and antibodies in the sera of the

diseased individual or demonstrate a high incidence of the virus in spontaneous tumors. These criteria are not enough to implicate a specific virus as the cause of the malignant disease in humans. The virus must be isolated as a productive virus and shown to have oncogenic capacity.

Viruses as the Etiology of Cancer

The introduction of embryonated chicken egg techniques by Woodruff and Goodpasture (1931) proved useful in the isolation of poxviruses and the viruses of influenza, herpes simplex, mumps and several other diseases. It became apparent, however, that techniques employing egg and animal inoculations were at times inadequate for the isolation and identification of many agents known to cause disease.

The cell culture techniques introduced by Weller *et al.* (1949) and Robbins *et al.* (1951) offered a new approach to the study of viruses. The essential elements for virus isolation and propagation consist of living cells and a physiologic media for their support. Cells cultivated *in vitro* have been derived from adult or embryonic tissues of primates and nonprimates, and many of these cultures have been propagated continuously for years. The virus susceptibility of discontinuously cultivated cell lines may be found to vary from the characteristics shown by continuously cultivated cells or from the same tissue when infected *in vivo* (Sheffield and Churcher, 1957). The variation in resistance makes it essential to select susceptible cells for cultivation of a given virus. For human viruses of unknown identity it is necessary to use cell cultures of different types, preferably embryonic tissue from the natural host or one closely related.

A number of agents associated with human diseases, e.g., poliovirus and varicella, propagate well in continuous and discontinuous cells (Enders et al., 1949; Weller et al., 1949) but do not propagate well in primary cultures derived from lower animals. Subsequently, investigations have demonstrated the capacity of human and other primate cells to support the growth of viruses that behave in an analogous manner (Ross and Syverton, 1957). Many times agents pathogenic for lower animals, which do not multiply in the cells of unrelated animals, can be cultivated easily in the cells of the same species, e.g., canine hepatitis (Fieldsteel and Emery, 1954).

One major difficulty in using cell cultures for virus isolation has been that agents unrelated to the disease syndrome may be recovered as a result of the presence of a passenger virus in the human tissue or through the activation of latent viruses in the animals or cells used for passage (Rivers, 1937). Another source of extraneous virus is serum used to supplement the cell culture media (Kniazeff and Rimer, 1967). Extraneous virus may produce cytopathogenic changes of cells in tissue culture, making it necessary to rule out known viruses that produce these same changes. The extraneous virus may appear as an inapparent infection or as a nonproductive virus in the cells. Frequent demonstration of viruses, whether passengers or contaminants, from human specimens including neoplastic tissue justifies further investigation of their relationship to human illness or malignant disease.

Certain factors are important in establishing an association between a newly isolated virus and a specific human illness. The critical criteria as summarized by Huebner (1959) can be stated as follows:

1. The virus must be a real entity established by animal or tissue culture passage.

2. It must be shown to have originated from humans.

3. It must evoke an antibody response in the human host.

4. It should consistently be associated with the same illness.

5. The virus should produce a predictable illness in volunteers.

6. Epidemiologic studies should substantiate evidence of association between virus infection and human illness.

Simple serologic procedures are available for routine laboratory investigation of many of the recognizable infectious viruses. In addition to assisting in the identification of isolated viruses, these serologic procedures can be used to confirm an isolate's relationship to a specific disease, or possibly to rule out extraneous viruses. When the demonstration of antibody increase, or exceptionally high titer during convalescence, can be shown, it is taken as valid evidence for association between the viral agent and the disease (Lennette, 1956).

Studies on polyoma virus demonstrated that a virus producing necrotizing lesions also possesses the capacity for inducing tumors in mice under special circumstances (Vogt and Dulbecco, 1960). This virus then disappears as an infectious agent from the tumor it induced. Certain human necrotizing viruses also possess a tumor inducing capacity, as was demonstrated by the induction of tumors in newborn hamsters with preparations of adenovirus type 12 (Trennin et al., 1962) and adenovirus 18 (Huebner et al., 1962). Subsequently, several other adenoviruses were demonstrated to possess oncogenesis (Pereira et al., 1965; Huebner et al., 1965; Girardi et al., 1964). The tumor induction caused by

adenoviruses, like polyoma virus induced tumors, was followed by the disappearance of infectious virus from the tumor. Virus specific non-structural antigens (T antigens) were produced in these cells as demonstrated by complement fixation tests (Hoggan et al., 1965; Huebner et al., 1963). The presence of these viral antigens suggested that the genome of the infecting adenovirus remained in the cell. Fujinaga and Green (1966) demonstrated by DNA-DNA homology experiments that the viral genome was incorporated as part of the genetic material of the host cell.

African Green monkey kidney cells infected with adenoviruses contained T antigen, but synthesis of adenovirus capsid antigen (V antigen) or infectious virus was absent. The aborted adenovirus growth cycle was completed with the release of infectious virus when papovirus SV-40 was concomitantly added. The virus released from cells infected with these two viruses possessed genotypic characteristics of either SV-40 or adenovirus but appeared phenotypically the same, since all viruses had an adenovirus capsid (Boege et al., 1966).

Viral genome recovery similar to the adenovirus SV-40 type has been demonstrated in mice following the discovery of a high mouse leukemia strain virus by Gross (1951). Other strains of murine leukemia virus were subsequently described (Moloney, 1964). These viruses represented a group of antigenically distinct agents capable of producing malignant diseases in mice (Old and Boyse, 1965). Examination of virus induced tumors of various types in mice and rats showed typical C-type particles budding from plasma membranes (Dalton et al., 1961). The particles were also revealed in cultured mouse embryo fibroblasts (MEF) infected with the leukemia viruses. These cultured cells were found to produce an

antigen that could be detected by complement fixation techniques (Hartly et al., 1965).

Huebner et al. (1965) found that murine sarcoma virus induced tumors in hamsters. The cells of these tumors contained the sarcoma virus genome but not infectious sarcoma or leukemia virus and no complement fixing antigens of the leukemia virus. The defective leukemia virus genome could be rescued from mixed cultures of hamster tumor cells and the normal MEF cells by addition of MLV. The resultant infectious sarcoma virus possessed the phenotypic expression of the MLV employed in the rescue, indicating that the sarcoma virus genome acquired the coat material synthesized by the MLV.

An analogous system was found with the defective Bryan strain of Rous sarcoma virus (RSV) which induced tumors in hamsters (Sarma et al., 1964). When tumor cells were propagated in mixed cultures of chicken embryo fibroblasts (CEF) the noninfectious RSV genome was transferred to the CEF cells. Superinfection of the mixed culture with avian leukosis virus resulted in the release of fully infectious RSV. When the uninfected mixed cultures were implanted into the wing web of leukosis free chickens, virus free sarcomas having the avian karotype were produced. These sarcoma cells, grown in tissue culture, contained no infectious virus, but with the addition of leukosis virus, infecting RSV was released.

The finding in humans of C-type particles similar in appearance to the murine and avian particles and the understanding that the virus complexes producing leukemia and sarcomas in mice do not differ greatly from the virus complexes producing lymphomatosis in chickens have led

to the assumption that leukemia in mammals may also be of viral origin. Tumor inducing ability is not limited to the RNA viruses demonstrating C-type particles in the murine and avian tumor cells, as shown by the polyoma and adenoviruses. Members of the herpes virus group have been found in tumor cells from leopard frogs with Lucké carcinoma (Fawcett, 1956) and lymphoid tumor cells from chickens with Marek's disease (Biggs and Payne, 1967).

Marek's disease is characterized by a proliferative involvement of the nerves and/or lymphoid tumors. The herpes virus associated with the disease was found in tumors, nerves, whole blood and many other areas of the body (Biggs and Payne, 1963). Infectivity of the virus was associated only with the cell containing fractions of various organs. Strains of Marek's disease have been transmitted through over 40 _in vivo_ passages (Biggs and Payne, 1963). It can be spread by direct and indirect contact and can be disseminated by the air-borne route suggesting that the agent was in the excretions and/or secretions of the infected chicken.

Cultured kidney cells of normal and infected chickens when used as recipients of various cell inocula demonstrated cytopathogenic effects (CPE) after 7-10 days in culture (Churchill and Biggs, 1967). The CPE appeared as small round plaques of cells having 1, 2 or 3 nuclei and occasionally Cowdry type A intranuclear inclusions were seen. The CPE was inhibited by DNA inhibitors. Chickens inoculated with tissue culture grown cells showing CPE produced specific lesions of Marek's disease.

Sections of cell culture showing CPE when examined by electron microscopy were revealed to contain herpes particles. These herpes particles were not separable from the tissue culture cells in an infectious form, suggesting that the physical techniques used to separate the virus from the cells readily inactivated the labile virus. The disease syndrome could be transmitted only by inoculating cells or cell debris.

Attempts to show a causal relationship between herpes viruses and human malignant diseases have entailed the isolation of herpes viruses from diseased tissue of individuals with tumors and then demonstrating antibodies against these viruses. Herpes simplex virus has been isolated from many patients with leukemia and lymphoma (Cleever et al., 1965), including patients with Burkitt's lymphoma (Simons and Ross, 1965). Herpes zooster manifests itself frequently as chicken pox in children with acute leukemia possibly because both diseases have a peak incidence in childhood (Bodey et al., 1965). Cytamegalovirus, another member of the herpes group, has also been detected in leukemia patients (Bodey et al., 1965; Duvall et al., 1966). Antibodies against these viruses have often been found in the blood stream of the patients from which the virus was isolated, but no correlation has thus far been shown with any malignant disease. The association of the herpes viruses to leukemia may be due to the debilitated state of the patient.

Nonproductive herpes-like particles have been revealed by electron microscopy in cultured neoplastic cells from patients with leukemia (Grace et al., 1965), lymphoma (O'Connor and Rabson, 1965), and in cells from Burkitt's lymphoma (Epstein et al., 1964; Stewart et al., 1965;

Toshima et al., 1967). These particles were found more frequently in cultured cells from patients with hematological disorders than in cells from patients with other diseases (Ames et al., 1967). These findings have stimulated a renewed interest in the herpes virus particles in cultured human cells. The herpes particle seen in the cultured Burkitt lymphoma cells has been of particular interest because of the abundance of these particles and the possibility of an etiologic relationship to the lymphoma.

Burkitt (1958) first described the disease as one which primarily affects children and is characterized by rapidly growing tumors located in the region of the mandible, which frequently metastasizes to the abdominal organs. This malignancy is fatal unless chemotherapy is employed, after which time recovery can take place (Burkitt, 1958). It was thought to exist in a limited geographic area in Africa or be confined solely to tropical regions. It should be emphasized that before a specific virus was associated with the Burkitt lymphoma, i.e., the herpes particle revealed by the electron microscope, the lymphoma appeared as an infectious process possibly a virus borne by an arthropod vector. Patients diagnosed as having Burkitt lymphoma have subsequently been found in other parts of the world, including America, Great Britain, New Guinea, and elsewhere (ten Seldam et al., 1966; Rowe and Johnson, 1964; O'Connor et al., 1965).

Bras et al. (1965) and Lukes et al. (1966) described a canine lymphoma which in their opinion was histologically indistinguishable from the Burkitt tumors. However, investigators who have examined both canine and human tissue deny this (Van Pelt, personal communication).

Baskerville et al. (1966) found 11 dogs, 3 cats, 4 pigs and 6 cattle with tumors similar to the Burkitt tumor.

A number of investigations have successfully established in vitro cultured cells from Burkitt's tumors (Epstein et al., 1964; Epstein et al., 1966; Pulvertaft, 1964; Stewart et al., 1965; and others). These cells grew in clusters of round lymphoma cells floating in the supernatant fluids. The clusters varied greatly, ranging in clumps of a few cells to a large aggregate made up of hundreds of cells. The individual cells were large, round and uniform.

Electron microscopy of the cultured cells disclosed virus-like particles originating in the nucleus (Toshima et al., 1967). The virus underwent substantial maturation, possibly resulting in damage to the nucleus and its membrane. If the virus acquired an outer coat it did so by budding through the nuclear membrane. Frequently the virus was extruded from the nucleus through a damaged membrane without obtaining an outer coat. The mature virus emerged from the cell by a process of budding through the cytoplasmic membrane.

Biologic activity was thought to be associated with the particles observed in these cells (Henle and Henle, 1965). Exposure of an intact cell line derived from Burkitt's lymphoma induced resistance to vesicular stomatitis virus in human embryonic kidney, human amnion, human diploid and green monkey kidney cells employed as feeder layers. The protective principle complied with the present criteria for interferon, suggesting that Burkitt's lymphoma cells in culture were infected with a latent virus.

Cultured Burkitt lymphoma cells, examined by the indirect immunofluorescent antibody technique, using sera from patients with Burkitt's lymphoma, or sera from American individuals whether healthy donors or patients suffering from a variety of illnesses, led to brilliant staining of a small number (1-5%) of the cells (Henle and Henle, 1966a). Attempts to relate the staining to known viral antigens failed to implicate herpes simplex, cytomegalovirus, varicells, reoviruses types 1, 2 and 3 and several other viruses. Examination of samples of Burkitt lymphoma cells in culture by the fluorescent test and electron microscopy, in parallel, showed three times fewer cells fluorescing than containing virus. Fluorescent positive cells were found to contain herpes-like particles when examined by the electron microscope, while nonfluorescing cells did not contain virus (Epstein and Anchong, 1968).

Sera from normal and leukemic individuals were found to contain complement fixing antibodies to partially purified antigens prepared from cultured Burkitt lymphoma cells (Henle et al., 1968; Gerber, 1968). Species of nonhuman primates possessed a high incidence of complement fixing antibodies to the purified antigen.

Patients with infectious mononucleosis regularly developed complement fixing and fluorescing antibodies to the herpes-type virus or antigens of that virus. The antibodies were found to persist for many years and were not distinct from heterophil antibodies, suggesting that the herpes virus or the antigens it stimulated are related to the agent causing infectious mononucleosis (Henle et al., 1968; Gerber, 1968).

Attempts to propagate these herpes-like viruses in cultures of normal human or animal cells have failed (Rabson et al., 1966; Epstein

et al., 1964). Stewart (1965) reported the successful passage of herpes-like viruses in the brains of newborn hamsters with a resultant progressive encephalytic syndrome ending in death but could not cultivate the virus in vitro. The infected brain cells could not be shown to contain herpes particles. Grace (personal communication) was able to transfer virus from cultured Burkitt cells to suspension cultures of human leukocytes but could not establish a necrotizing infection in monolayer cultures of cells or to separate the virus as an infectious agent free of cellular material.

It has not been established whether these herpes viruses are the etiologic agents of Burkitt's lymphoma, a passenger in the tumor tissue or a contaminant from a common source. Another question to be answered is whether these herpes-like particles are defective and require a helper virus similar to the murine and avian virus complexes. These questions cannot be answered until the virus has been isolated from the cultured cells as an infectious agent and its characteristics studied.

Isolation of a Virus from Burkitt Lymphoma Cells

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Canine thymus cells were used to isolate a virus (MDH-1) from Burkitt lymphoma cultures superinfected with Moloney sarcoma virus. Morphological detail, the pattern of cell degeneration and inclusion bodies produced suggest that MDH-1 virus belongs in the herpes virus group. Complete details of this isolation and description of the virus are presented in publication form on the following pages.

Preliminary Communication
reprinted from THE LANCET, *June* 24, 1967, *pp.* 1358-1359

ISOLATION OF A VIRUS FROM BURKITT LYMPHOMA CELLS

Summary Canine thymus cells were used to isolate a virus (MDH-1) from Burkitt lymphoma cultures superinfected with Moloney sarcoma virus. Morphological detail, the pattern of cell degeneration, and inclusion bodies produced suggest that MDH-1 virus belongs in the herpesvirus group. Electron-microscopic and serological examination, however, indicates that it is unlike any virus previously described.

INTRODUCTION

SEVERAL workers have discussed the likelihood of a virus ætiology for Burkitt lymphoma on the basis of epidemiological findings and the demonstration by electron microscopy of herpes-like virus particles in cell cultures derived from several of these tumours.[1-4] Repeated attempts to grow these herpes-like entities in other cell cultures have been made in several laboratories, without success. Viral agents, however, have been isolated from Burkitt lymphoma tissues.[3-6] These include *Herpesvirus hominis* and reoviruses. Hitherto no association between these agents and the virus particles seen in cell cultures derived from Burkitt tumours has been demonstrated to date.

This paper reports the isolation from P3-J Burkitt cells of a herpes-like virus (MDH-1) which, so far as we know, has not previously been described. It is not neutralised by, nor does it fix complement in the presence of, antiserum to *Herpesvirus hominis*.

MATERIALS AND METHODS

Cell Cultures

Stock cultures of the P3-J line (Jiyoye) of Burkitt lymphoma cells (obtained from Pfizer Laboratories) were grown in 32-oz. prescription bottles containing 100 ml. of Eagle's minimum essential medium plus 20% unheated fœtal calf serum. New cultures were started with 5×10^5 cells per ml., were fed by replacing 50% of the medium at 3–4 days, and were usually split weekly. This procedure resulted in a doubling-time of about a week.

Canine thymus (CThy) and canine kidney (C.K.) cells were obtained from puppies 2–6 weeks of age by the usual trypsinisation procedure and were grown in 32-oz. prescription bottles. Bottles seeded with 10^8 CThy cells in 50 ml. of medium 199 plus 20% unheated fœtal calf serum developed a confluent cell sheet in 7–10 days. These primary cultures were maintained with medium 199 plus 5% unheated fœtal calf serum until needed. Bottles seeded with $10–15 \times 10^6$ C.K. cells in 50 ml. of Eagle's basal medium (B.M.E.) plus 20% unheated fœtal calf serum developed a confluent cell sheet in 3–5 days. These cultures were maintained with B.M.E. plus 5% unheated fœtal calf serum. Secondary cultures of CThy and C.K. were produced from cells trypsinised from the primary cultures. To permit an extended period of observation for possible adventitious virus cytopathogenic effect (C.P.E.) in the primary cells, all virus studies were done in secondary cultures.

Human embryonic kidney (H.E.K.) cells and WI-38 human diploid fibroblasts were purchased in screw-cap tubes from Microbiological Associates, Inc. The medium in these tubes was replaced with B.M.E. plus 5% unheated fœtal calf serum upon delivery and was usually used within 1–3 days of delivery.

All cell cultures were grown and maintained at 37°C and were held at 35°C after inoculation.

Virus Inoculations

Three-oz. prescription bottles containing $2·5 \times 10^6$ P3-J cells were inoculated with 17 known viruses representing the herpesvirus, adenovirus, murine leukemia, reovirus, myxovirus, poxvirus, and papova virus groups. Samples of cells and medium were removed from the superinfected P3-J cultures and were inoculated directly into tube cultures of H.E.K., WI-38, C.K., and CThy cells at intervals of 7–10 days post-inoculation. These cells were observed for C.P.E. until spontaneous cell degeneration occurred. Viruses recovered from this procedure were compared with the original superinfecting virus by serum neutralisation and examination of host range, or other properties, according to the nature of the virus in question.

RESULTS

Canine thymus cells inoculated with the 10-day sample of P3-J cells superinfected with Moloney sarcoma virus showed C.P.E. within 48 hours after inoculation. Further comment will be limited to the virus, MDH-1, isolated in this CThy culture. 5 days after inoculation, when cell degeneration was nearly complete, cells and fluid from the CThy cultures were passed to fresh cultures of H.E.K., WI-38, C.K., and CThy. Within 48 hours C.P.E. was again observed in CThy and also appeared in H.E.K. Degeneration in both cell lines was complete in 5 days. The virus has been carried through eight serial passages in H.E.K. and six serial passages in CThy with no apparent change in the pattern of C.P.E. Examination of infected cell cultures for the presence of mycoplasma was negative.

Control cultures of P3-J cells carried in parallel with superinfected cultures did not produce any C.P.E. when inoculated into H.E.K., WI-38, C.K., and CThy.

Moloney leukemia virus was inoculated into P3-J cells and studied in the same manner as Moloney sarcoma virus with negative results. Moloney sarcoma virus was inoculated directly into H.E.K., C.K., CThy, and canine embryo cells. No C.P.E. was observed in these cell cultures after primary inoculation or after several blind passages in H.E.K. and CThy.

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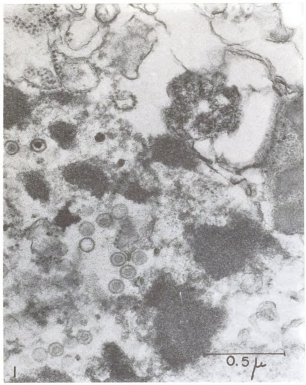

Fig. 1—Naked virus particles: thin section of nucleus of infected canine thymus cell.

Glutaraldehyde/osmic-acid fixation. Uranyl-acetate/lead-plumbite staining.

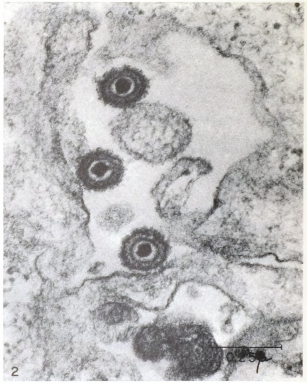

Fig. 2—Membrane-enveloped virus particles: thin section of cytoplasm of infected canine thymus cell.

Glutaraldehyde/osmic-acid fixation. Uranyl-acetate/lead-plumbite staining.

The MDH-1 virus has the following characteristics: It produces a herpes-like C.P.E. in H.E.K., HeLa, continuous human amnion, Chang liver, C.K., CThy, canine embryo, and African green monkey kidney cells. No C.P.E. has been observed, however, in WI-38, human embryonic lung, HEp2, rhesus monkey kidney, or mouse embryo cells from Swiss-Webster mice, nor have any lesions been observed in these mice inoculated when 2 days old and observed for 32 days.

Examination of infected canine thymus cells by light microscopy revealed intranuclear inclusions similar to those seen in cells infected with viruses of the herpes group. Examination of these cells by electron microscopy demonstrated herpes-like virus particles within the nucleus that measured 70–80 mμ in diameter (fig. 1). These intranuclear particles are enveloped by the nuclear membrane upon release from the nucleus into the cytoplasm of the cell. The resulting particles are 110–160 mμ in diameter (fig. 2). The size of these herpes-like particles is within the range of those previously described as being associated with the Burkitt lymphoma cells.[2 4 7] Electron-microscopic studies are currently in progress to examine the host-cell virus relationship at the ultrastructural level.

Only limited serologic studies have been conducted to date with the MDH-1 virus. The virus is not neutralised by antisera against reovirus types 1, 2, and 3, *Herpesvirus hominis*, or Moloney sarcoma virus. The virus failed to fix complement in the presence of adenovirus group antiserum or *Herpesvirus hominis* antiserum.

DISCUSSION

The results of these studies indicate that a previously undescribed virus has been recovered from the Burkitt lymphoma cell cultures superinfected with Moloney sarcoma virus. The dissimilarity of host range and the morphology of MDH-1 indicate that it is neither Moloney sarcoma virus nor Moloney lymphoma virus. The morphology of the MDH-1 virus particle, as well as the nature of the intranuclear inclusions and the cell degeneration produced, indicate that it is a member of the herpes-virus group. The results from the serological tests suggest that it is not *Herpesvirus hominis*.

The significance of this isolation and its association, if any, with Burkitt lymphoma cannot be assessed without further study. The possibility of MDH-1 being a hybrid virus or a latent virus previously unobserved in the cell culture system must be considered. The immunological, morphological, biological, and physical characteristics of the virus and its association or lack of association with human neoplasia or other disease is under investigation.

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Characterization of a Herpes-Like Virus Recovered from Burkitt Lymphoma Cells (P3-J) and Propagated in Dog Thymus Cells. I. An Electron Microscopic Study

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Characterization of a Herpes-Like Virus Recovered From Burkitt Lymphoma Cells (P3-J) and Propagated in Dog Thymus Cells. I. An Electron Microscope Study [1,2]

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SUMMARY—Electron microscopic studies of a herpes-like virus recovered from the P3-J line of Burkitt lymphoma cells superinfected with Moloney mouse sarcoma virus are described. The virus was initially isolated in newborn dog thymus cells in culture and demonstrated a productive type of infection as reported by Mitchell, Anderson, Bowles, and Hinz. To date all attempts to identify this agent antigenically have failed. Detailed ultrastructural studies of the developmental stages at different intervals after infection in dog thymus cells revealed the following: 1) The virus followed typical morphologic patterns associated with herpesviruses; 2) the particle size of both naked (immature) and membrane-associated virus was on the average within the size range of herpes simplex viruses; 3) electron-dense particles averaging 35mμ in diameter formed in the cytoplasm 4–8 hours after infection and appeared to be in a juxtanuclear membrane position; 4) similar dense particles appeared within the nucleus 6–8 hours after infection and concurrently with naked virus particle formation; 5) naked herpes-like virus particles were formed, approximately 8–12 hours after infection, in what appeared to be two ways: a) as a very dense, sharply limited viroplasmic-like substance within the nucleus and b) as single particles randomly distributed throughout a homogeneous nucleoplasm; 6) after naked virus production, the formation of membrane-associated virus was like that previously described for herpes simplex virus. The antigenic nature and oncogenic capacity of this agent are currently under investigation.—J Nat Cancer Inst 40: 477–489, 1968.

MANY CONTINUOUS cell cultures have been derived from Burkitt lymphoma tissues, other solid tumors, and buffy coat cells. At least 28 of these cultures have been shown by electron microscopy

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[5] A portion of this investigation was carried on as a partial fulfillment for the requirements of the Ph.D. degree in the Department of Microbiology and Public Health, Michigan State University.

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to contain virus-like particles morphologically similar to the herpesvirus group (unpublished report, National Cancer Institute, Aug. 1966). Herpes-like particles as seen by electron microscopy in Burkitt tumor cells have been described by Epstein *et al.* (*1–3*), Stewart *et al.* (*4*), O'Conor and Rabson (*5*), Toshima *et al.* (*6*), Toplin and Schidlovsky (*7*), and others. Although there have been several unpublished and published (*8, 9*) reports of the isolation of herpes simplex virus directly from the Burkitt lymphoma tumor, no antigenic association has been reported between any known virus and the virus particles commonly found in continual cultures of Burkitt lymphoma cells (*10*).

Attempts to recover the herpes-like virus from Burkitt lymphoma cell culture in the authors' laboratories have been based in part on the assumption that interaction with another virus may be required, as is the case for the defective Bryan strain of Rous sarcoma virus (*11*) and the defective Moloney sarcoma virus (*12*).

Cultures of the P3-J (Jiyoye) line of Burkitt lymphoma cells were superinfected with known viruses of human or animal origin. By electron microscopy, this line of Burkitt cells in culture had previously been shown to contain herpes-like virus particles, but all attempts had failed to propagate this agent in other cell culture systems. At intervals of 7–10 days, media and cells from the P3-J cultures were transferred to monolayer cell cultures of human and canine origin. Viruses recovered from the P3-J cultures were compared with the original superinfecting virus by a study of the host range, neutralization by homologous antisera, and other biologic properties. One virus, MDH-1, recovered from a P3-J culture superinfected with Moloney sarcoma virus proved quite different from the superinfecting virus. It has been tentatively identified as belonging to the herpesvirus group on the basis of morphology and biologic activity (*13*). To date, the MDH-1 virus has not been neutralized by prototype herpesvirus antisera. A detailed study of its developmental stages at the ultrastructural level is reported herein.

MATERIALS AND METHODS

Cell cultures.—Stock cultures of the P3-J line of Burkitt lymphoma cells (kindly supplied by Chas. Pfizer & Co., Maywood, N.J.) were grown in 32-ounce prescription bottles containing 100 ml of Eagle's minimal essential medium plus 20% unheated fetal calf serum. New cultures were started with 5×10^5 cells/ml, were fed by 50% of the medium being replaced at 3 or 4 days, and passed weekly with a 1:1 dilution into 2 bottles. This procedure resulted in a doubling time of about 1 week.

Thymus cells were obtained from young dogs, 2–6 weeks old, by the usual trypsinization procedure, and grown in 32-ounce prescription bottles. Bottles, seeded with 10^8 cells in 50 ml of medium 199 plus 20% fetal calf serum, contained a confluent cell sheet in 7–10 days. These primary cultures were maintained with medium 199 plus 5% unheated fetal calf serum until needed.

Secondary canine thymus cultures for virus inoculation were obtained by trypsinization of the primary cultures. Three-ounce prescription bottles were seeded with 2×10^6 cells in 8 ml of medium. Growth and maintenance media for the secondary cultures were the same as those used for the primary cultures.

Virus inoculation.—Three-ounce prescription bottles containing confluent sheets of canine thymus cells were washed with basic salt solution (BSS) and then inoculated with 1 ml of culture fluid containing 10^4 TCID50 of MDH-1 virus. The inoculated cultures were incubated for 30 minutes at 37°C before the maintenance medium was replaced. Cells for electron microscopic study were harvested from the inoculated cultures at ½, 4, 6, 8, 12, 18, 24, 48, 72, and 96 hours after infection.

Electron microscopy.—Cells in culture to be fixed and blocked for viewing by electron microscopy were washed twice with BSS and mechanically removed from the glass surface with a rubber policeman. The cells were resuspended in BSS and pelletized by centrifugation at approximately $80 \times g$. Pelletized cells were cut into fragments and quickly placed in cool 3.5% glutaraldehyde solution (*14*), buffered at *p*H 7.3. After 12 hours, the cell fragments were washed in Sorensen's buffer solution and fixed in 1% osmium tetroxide for 1 hour (*15*). The cells were then dehydrated in increased concentrations of ethanol followed by

propylene oxide, propylene oxide-Epon, and embedding in Epon 812 (*16*). Ultrathin sections were cut with diamond knives, placed on 300-mesh copper grids coated with carbon-stabilized Formvar and double-stained with uranyl acetate and lead citrate (*17*). All sections were examined with a Siemens Elmiskop 1-A electron microscope.

RESULTS

Cells cultivated *in vitro* may exhibit ultrastructural changes common to both virus-infected and noninfected cells. The cellular alterations described in this communication are exclusively those which were thought to be virus-associated and did not resemble degenerative changes due to *in vitro* cultivation and/or age.

Usually dog thymus cells cultured *in vitro* as controls appeared normal for 48–96 hours. The ground substance of the cytoplasm during this stage also appeared normal. The nucleus with its membrane and organized contents did not show any visible degenerative change.

Ultrastructural changes in virus-infected cells, however, were seen beginning 4–8 hours after initial infection. Few cellular changes were seen before this time. Cytoplasmic changes occurred first in approximately 25% of the cells in the form of uniform, electron-dense particles approximately 35mμ in diameter. The number of ribosomes increased slightly, and the mitochondria appeared unaltered. Although some of these particles seemed to form near or adjacent to the surface of cytoplasmic membranes, most of them had no visibly defined point of origin and were not associated with any particular cytoplasmic structure. Concurrent with electron-dense particle formation was a moderate increase in vacuole formation within the cytoplasm.

Electron-opaque cytoplasm particles seemed to migrate to a juxtanuclear membrane position and to arrange themselves in contact with the outer nuclear membrane (fig. 1). In those areas where apparent contact occurred, the nuclear membrane seemed to partially lose its integrity (figs. 2 and 3), and a limited number of particles appeared to be entering the nucleus or similar intranuclear particles seemed to form in that area. Nuclear alterations first appeared as irregular distributions of chromatin. In some nuclei, a few dense clumps of chromatin marginated at the nuclear membrane with the formation of more intranuclear electron-dense particles (fig. 4). The inner nuclear membrane at this time began to show some signs of thickening.

From 8–18 hours following infection, naked (immature) virus particles seemed to be forming in the nucleus in one of two ways.

1) Most commonly, numerous virus particles formed in a very dense, sharply defined viroplasmic-like substance. This substance was not contained in a membrane and the clusters of viruses formed were not oriented in any defined geometric pattern. The viruses did, however, form close to one another. The nucleus appeared partially empty after naked virus formation, with most of the chromatin marginated (figs. 5, 6, and 8). The inner portion of the nuclear membrane showed a marked increase in thickness and density. The immature virus particles ranged from 95–110 mμ in diameter and contained a dense pleomorphic nucleoid approximately 35–40 mμ in diameter (figs. 14 and 15). During this period the ribosomes of the infected cells became more numerous and electron opaque, and vacuolization of the cytoplasm was markedly increased, while some mitochondria seemed to be degenerating.

2) Sometimes the viruses were distributed randomly throughout the nucleoplasm. When particles formed in this manner, there were fewer cellular changes seen in both the nucleus and cytoplasm associated with virus formation. Chromatin margination was not as marked and the nucleoplasm seemed to be well distributed throughout the nucleus (figs. 7 and 9). During the 8- to 18-hour period, occasionally clusters of naked virus particles were in a matrix of nucleoplasm, but they were much less dense and did not have a sharply limited periphery (fig. 14).

When immature herpes-like virus particles were randomly distributed throughout the nucleus, they often acquired the inner nuclear membrane by budding into the cytoplasm (fig. 18). Intranuclear whorls of nuclear membrane extensions were also frequent (figs. 6 and 7). In some instances during this time those naked virus particles which were associated with a viroplasmic-like material seemed to be located adjacent to, but

outside, the periphery of the mass and gave rise to a "sunflower" appearance. Thus it is assumed that particles were being disassociated from this substance (fig. 10).

From 24–48 hours after infection, cells could be found supporting only immature virus formation and/or initiating the formation of double-membrane virus particles. The formation of double-membrane particles was the result of maturation of naked virus budding through cellular membranes, thus acquiring a covering from these particles as the virus passed through. This period of time seemed to be the transition period between immature and mature virus formation.

From 48–96 hours after infection of the canine thymus cells, the most common finding was the membrane-associated viruses, *i.e.*, viruses thought to be associated with nuclear membrane material. These particles were found randomly throughout the cytoplasm and nucleus (fig. 11). When membrane-associated viruses were in the cytoplasm, they were double-membrane particles forming intravesicularly (figs. 12, 13, and 16). The host cell at this stage of virus development was beginning to show signs of marked degeneration, as evidenced by many single- and double-membrane vacuoles in the cytoplasm. Some of the latter may have been derived from swollen mitochondria which could still be recognized by remnants of cristae. At this stage, long, electron-dense, altered spindle tubules were common in the cytoplasm (fig. 6).

The double-membrane viruses measured from 170–195 mμ, whereas the triple-membrane viruses measured from 185–200 mμ. When double- and triple-membrane viruses were seen, cellular destruction was evident, and frequently nuclear and cytoplasmic material was arranged in such a fashion that all evidence of an intact cell had disappeared. During this time extracellular mature virus particles were seen. Since control cells were markedly altered at 96 hours, it was difficult to estimate by electron microscopy what percentage of the total cells in culture supported virus replication at 96 hours following infection. It was estimated that 50–65% of the total cells were infected. The highest titer was reached between 72–96 hours and at the time of this writing did not exceed 10^5 TCID50 in dog thymus and cells from 33-day-old dog embryos.

DISCUSSION

Since the association of a herpesvirus with Burkitt lymphoma has so frequently been reported, the finding of a productive infection in newborn dog thymus cells by a herpes-like virus recovered from P3-J Burkitt lymphoma cells superinfected with Moloney's mouse sarcoma virus is indeed significant. In addition, the herpesvirus, so common in Burkitt cell lines, has not been identified immunologically as belonging to the presently known herpesviruses (*13*, *18*, *19*). Since the work of Epstein *et al.* (*1–3*) indicates that the herpesvirus of Burkitt lymphoma resembles herpes simplex virus, with some exceptions (*20*), it was relevant to observe at the ultrastructural level the various stages of development of this isolated virus at preselected intervals. Until now such a study of a Burkitt cell-associated herpesvirus propagated in a normal heterogeneous cell culture system has not been reported. Therefore, it is of further relevance to compare and contrast the various stages of development of the MDH-1 virus with those of the herpes simplex virus and herpes-like viruses common in the Burkitt lymphoma cells.

The findings in this laboratory have shown that electron-dense particles occur in the cytoplasm and nucleus before there is evidence of incomplete virus formation. Shipkey *et al.* (*21*) found similar electron-dense particles, resembling virus nucleoids and measuring 30–60 mμ in diameter, only in the nucleus in herpes simplex infections. The particles seemed to precede incomplete herpes simplex virus formation and were similar to those seen in the first stages of infection associated with the MDH-1 agent. The importance of MDH-1 virus-induced dense particles being consistently present within the cytoplasm, migrating and becoming associated with the outer nuclear membrane, is not known. Present studies are currently underway to determine if these particles are glycogen. In other types of dog cells in culture infected by virus, glycogen deposits usually do not take on such a uniform size as is demonstrated here. It is, however, of interest since this finding is common. During this time

chromatin margination is also seen and is consistent with herpes simplex virus formation.

Cell cultures infected with herpes simplex virus show convolutions of the nuclear membrane (*22*), reduplication of the membrane (*23*), and thickening of the nuclear membrane (*24*). These same alterations are seen in dog thymus and dog embryo cells infected with MDH-1 virus.

When the MDH-1 virus appears to form at random throughout the nucleus, degeneration of the intact cell is not as marked. Conversely, when naked virus particles are seen contained in the very dense, sharply delineated viroplasmic-like substance, the nucleus shows a marked increase in chromatin margination and seems to be devoid of nucleoplasmic substance. The reason for, or significance of, these two phenomena in naked virus particle formation is not known; however, the involvement of a different target cell type in dog thymus might be one possibility. In either event, the formation of naked virus particles is preceded by 35–40 mμ electron-dense particles appearing both in the cytoplasm and the nucleus. It seems very unlikely that viral DNA would first form in the cytoplasm and then migrate to the nucleus. The loss of the integrity of the nuclear membrane in different focal areas, which would allow cytoplasmic electron-dense particles to penetrate, is so frequent during the early stage of development that it is unlikely to be an artifact. However, the number of electron-dense particles in the nucleus following infection is so great that it is difficult to envision their being introduced from the cytoplasm. It is therefore assumed that most of these dense intranuclear particles are formed in the nucleus and could very well represent viral DNA.

Our studies suggest that naked virus particle formation begins as a single or double hexagonal shell and acquires its nucleoid at a later time, possibly from the inner membrane of the double-shelled naked particles. It is possible, however, that the single-shelled particles, devoid of a nucleoid, are incomplete and will never acquire a nucleoid.

It is common to see single-shelled particles attached to the inner nuclear membrane and budding through and into the cytoplasm in those cells where the naked virus particles are randomly distributed throughout the nucleoplasm. As reported by Toshima *et al.* (*6*), immature herpesvirus particles seen in the Burkitt cells most likely reach maturity by being discharged from a damaged nucleus into the cytoplasm. Maturation of the MDH-1 virus occurs at times in a similar fashion; however, budding from the nuclear membrane into the cytoplasm or nuclear membrane extensions within the nucleus have been seen occurring in cells which show minimal signs of nuclear damage (figs. 16 and 18). Our findings show that thickening of the inner nuclear membrane does not seem to be associated with naked particle attachment, which is consistent with the findings of Falke *et al.* (*22*).

The formation of double- and triple-membrane-associated virus takes place in direct association with nuclear membrane material. When this occurs in the cytoplasm, it is a function of the reduplication and/or proliferation of the nuclear membrane which has dissociated from the main body of the nucleus and becomes localized in the cytoplasm. It is common to see double-membrane-associated herpes-like viruses forming in the nucleus and cytoplasm simultaneously. The virus is released from the cell when the integrity of the cell is destroyed by virus infection.

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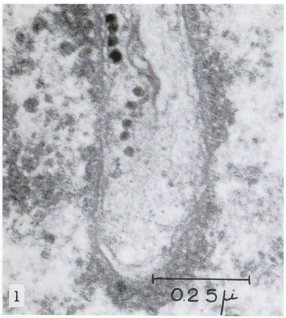
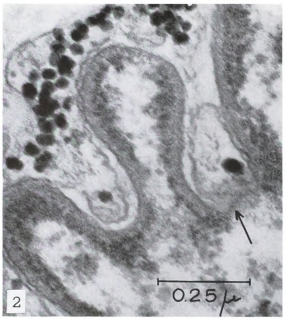
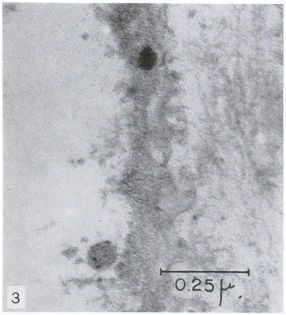






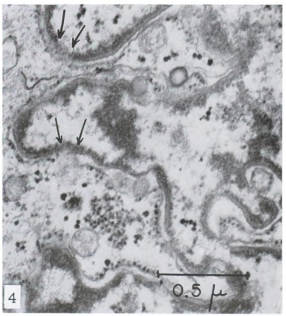


FIGURE 1.—Particles arranged between lamella of nuclear membrane, 6 hours after infection. × 110,000

FIGURE 2.—Electron-dense particles in a juxtanuclear membrane position. *Note* portion of cytoplasmic material containing particle detaching and partial loss of integrity of nuclear membrane in this area, 6 hours after infection. × 104,000

FIGURE 3.—Electron-opaque particle located within substance of inner lamella of nuclear envelope. × 96,000

FIGURE 4.—Electron-opaque particles in both cytoplasm and nucleus. *Note* apparent damage of nuclear envelope near area of concentrated intranuclear particles. × 50,000

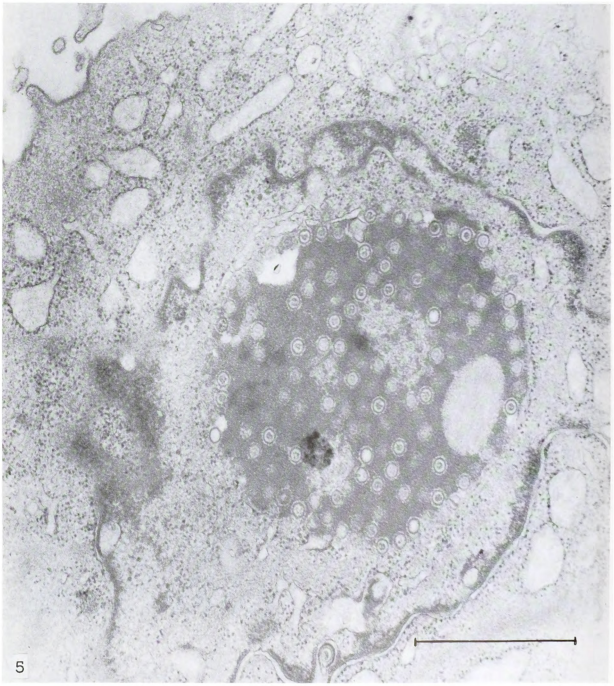
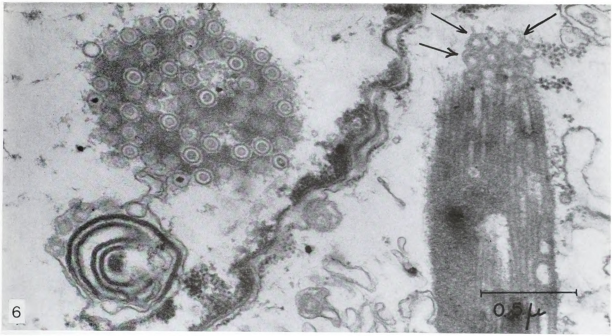
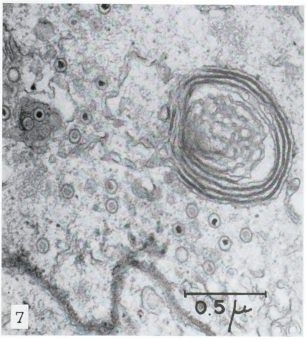


FIGURE 5.—Naked herpes-like virus particles forming in viroplasmic-like substance within nucleus. Associated with each maturing naked virus particle is a halo. Various developmental stages of formation of naked virus particles are represented, 18 hours after infection. × 43,000





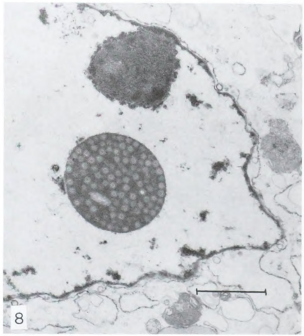
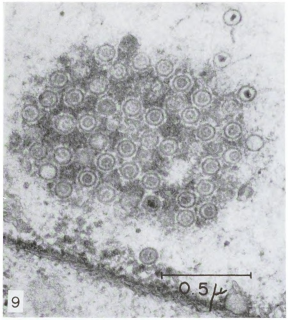
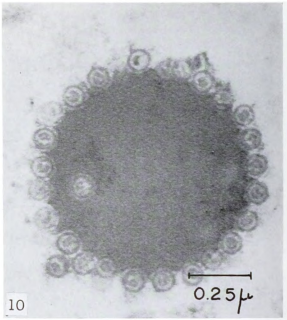


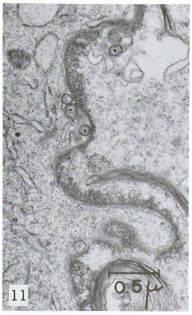
FIGURE 6.—Naked virus particles and whorls of nuclear membrane extending inside nucleus. *Note* intracytoplasmic fibers contain structures which are electron-opaque, altered spindle tubules. × 50,000

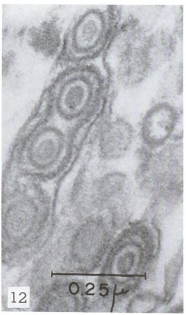
FIGURE 7.—Randomly distributed, single-shelled virus particles and nuclear membrane extensions occurring as whorls in nucleus, 18 hours after infection. × 44,000

FIGURE 8.—Single-shelled virus particles replicating in dense viroplasmic-like substance, 18 hours after infection. × 18,000









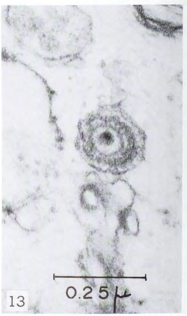


FIGURE 9.—Virus particles replicating in condensed nucleoplasm, 18 hours after infection. × 50,000

FIGURE 10.—Viroplasmic-like substance supporting virus replication at periphery of mass, producing a "sunflower" appearance. × 70,000

FIGURE 11.—Intranuclear and extranuclear double-membrane particles formed in vacuoles. × 26,000

FIGURE 12.—Virus particles contained in pods within cytoplasm. Double-membrane particles are formed by "pinching off" and carrying pod membrane with it. × 108,000

FIGURE 13.—Typical double-membrane particle contained in cytoplasm of cell, 48 hours after infection. × 104,000

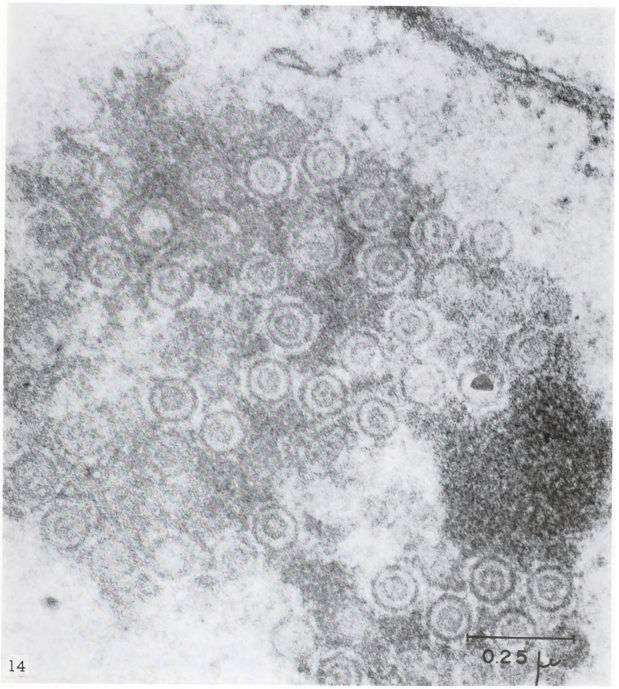
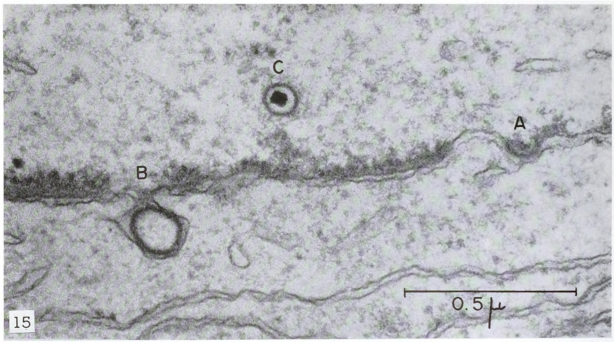


FIGURE 14.—Virus replicating in what appears to be condensed nucleoplasm. Most particles suggest concurrent formation of single shell and nucleoid. × 115,000



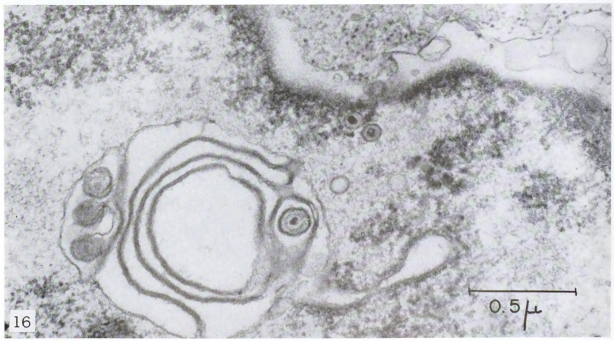
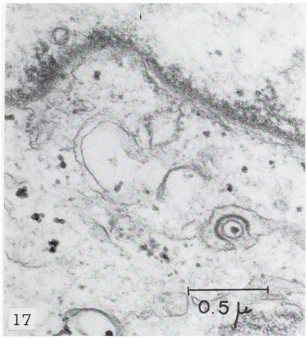
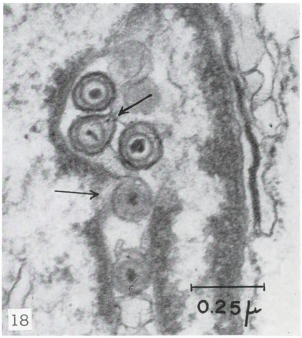
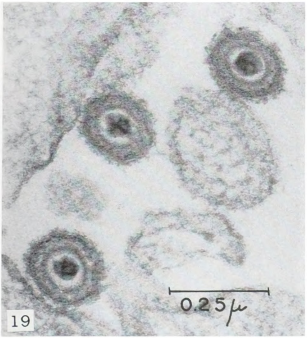


FIGURE 15.—Budding at nuclear membrane. (A) Beginning of budding process, but *note* absence of any single naked virus initiating the bud; (B) budding of particle devoid of nucleoid; and (C) single-shelled particle. × 92,000

FIGURE 16.—Double-membrane virus particles forming in whorls of nuclear membrane extensions inside nucleus. × 55,000







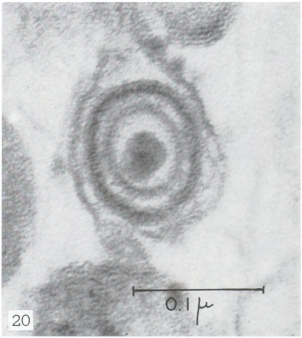


FIGURE 17.—Virus particle budding to form a double-membrane particle within cytoplasmic vacuole, 48 hours after infection. × 42,000

FIGURE 18.—Double-membrane virus particles budding from nuclear membrane into cytoplasm, 48 hours after infection. *Note* pleomorphic nucleoids. × 78,400

FIGURE 19.—Double-membrane intracytoplasmic particles formed in vacuole, 22 hours after infection. × 107,000

FIGURE 20.—Membrane-associated particle forming within cytoplasm, 72 hours after infection. × 180,000

Characterization of a Herpes-Like Virus Recovered from Burkitt Lymphoma Cells (P3-J) and Propagated in Dog Thymus Cells. II. Physical and Biological Studies

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Additional information concerning MDH-1 is required before the origin of the virus and its relation to the Burkitt lymphoma cells in culture can be established. Certain characteristics of MDH-1 virus were therefore determined. The detailed results of these efforts are presented on the following pages and appear in part as a manuscript submitted for publication to the Journal of the National Cancer Institute.

INTRODUCTION

A herpes-like virus has frequently been seen in solid tissues and established cells cultured from Burkitt's lymphoma (Epstein et al., 1965; Toshima et al., 1967; Epstein et al., 1964). One of the primary concerns has been to recover these agents and determine their oncogenic capacity and/or capability as well as their physical and biological characteristics.

Efforts have been made to establish a serological relationship of these virus-like particles to the original tumor (Gerber and Birch, 1967; Mayyasi et al., 1967; Henle and Henle, 1966a) and antibodies associated with other human diseases (Henle et al., 1968). Attempts at determining some of the physical characteristics have also been made with particles separated from the Burkitt cells (Toplin and Schidlovsky, 1966). These efforts have been limited, however, by the fact that agents under study were not infectious by the techniques employed.

The recovery of an infectious virus from the P3-J line of Burkitt's lymphoma after superinfection with Moloney Sarcoma virus (Mitchell et al., 1967) presented the first opportunity to investigate these herpes-like particles. Investigations to define this agent (designated MDH-1) in our laboratory have been under way since its recovery, and the results of our findings to date are reported below.

MATERIALS AND METHODS

Tissue Culture: Continuous cultures of Madin-Darby bovine kidney (MDBK) and canine kidney (MDCK), syrian hamster kidney BHK-21, clone of strain L mouse cells NCTC 929 (L-929), Chang Liver (Ch.L.) and HeLa (GEY) cells

(purchased from American Type culture collection, Rockville, Maryland) were propagated at 37° C in 32-ounce bottles with growth medium consisting of Hanks' minimal essential medium containing 10% inactivated fetal calf serum (HMEM-10). All media used in the study contained 100 μ/ml of penicillin and 100 micrograms/ml of streptomycin. Replicating cells were dispersed by a 0.25% solution of trypsin (Difco 1:250) or a trypsin-versene solution (Madin and Darby, 1958) for subculturing and were inoculated into bottles at a concentration of 4×10^6 cells/bottle. Cultures were fed one or two times each week and subpassaged once a week.

Cell culture tubes were prepared by seeding 1.5×10^6 cells in 1 ml of HMEM into 125 x 12 mm tubes and incubating at 37° C until confluent cell layers formed. The growth medium was decanted and 1 ml of Earle's MEM containing 5% inactivated fetal calf serum (EMEM-5) was added. The tubes were used the following day.

Primary cultures of dog embryo fibroblast cells (DEF) were obtained from cesarean derived 30-33 day old Beagle dog embryos. The embryos were washed in Hanks' balanced salt solution (HBSS) and minced into 3-5 mm pieces. The minced embryos were washed several times in HBSS and added to stirring flasks containing 0.25% trypsin in HBSS. Cells in suspension were removed from the trypsin solution every 15-20 minutes, pelletized by centrifugation at 2500 rpm in a PR-2 International centrifuge, head #253. The cells were seeded into 32-ounce bottles containing HMEM-10 at a concentration of 1×10^6 cells/ml. Cultures were fed 1-2 times weekly and were subpassaged when confluent monolayers formed.

Tubes or 32-ounce bottles containing primary cultures of human embryonic kidney cells (HEK) were purchased from Microbiological Associates,

Inc., Bethesda, Maryland. Upon receipt, fresh media consisting of EMEM-5 was added to the cultures and incubated at 37° C.

Virus: MDH-1 and infectious bovine rhinotracheitis (IBR) virus, Los Angeles strain, were prepared by inoculating confluent cultures of HEK or MDBK cells in 32-ounce bottles with 3 ml of virus titering 10^4-10^6 $TCID_{50}$/0.1 ml. Following adsorption for one hour at 37° C the cells were carefully washed twice with HBSS and 35-40 ml of EMEM-5 was added. Infected cultures were incubated at 37° C until cell destruction occurred (usually 3-4 days). The cultures were frozen and thawed twice and supernatant fluid containing virus was collected following centrifugation at 2500 rpm for 10 minutes in a model PR-2 International centrifuge, head #253. The supernatant containing the virus was ampuled in 1 ml aliquots, quick frozen using dry ice and alcohol and stored at -70° C.

Infectivity titrations were performed on the frozen stock at regular intervals in tubes containing MDBK cells. Serial 10-fold dilutions of the stock virus were prepared using HBSS. To each of three tubes 0.1 ml of the virus dilution was added and incubated at 37° C. The cultures were refed on the 3rd day and read for cytopathic effects (CPE) daily for 7 days. Infectivity titers were expressed as $\log_{10}$ tissue culture infective dose - 50 percent ($TCID_{50}$) per 0.1 ml. Titers were calculated using the procedure of Reed and Muench (1938). Using MDBK cells titers ranged from 10^6-10^7 $TCID_{50}$ per 0.1 ml of virus suspension.

MDH-1 and IBR viruses used in density gradient studies were concentrated from MDBK propagated stock virus preparations by centrifugation in 50 ml polycarbonate tubes at 33,000 rpm for 2 hours using a model B-35 International preparative ultracentrifuge, head #A-147. The

supernatant fluid was decanted and the virus pellet was resuspended in a volume of HMEM so that the original virus suspension was concentrated 100-fold. This suspension was further clarified at 5000 rpm in 12 ml polycarbonate tubes using the B-35 ultracentrifuge, head #SB-206. Virus particles from the P3-J line of Burkitt lymphoma cells were separated by three freeze-thaw cycles and then centrifuged at 2500 rpm in a PR-2 International centrifuge, head #253, for 20 minutes. The virus was concentrated in the same manner as MDH-1 and IBR. The viruses were ampuled in 1 ml aliquots and quick frozen at -70° C as indicated above.

Sera: Anti-MDH-1 virus serum was prepared in adult rabbits by a series of eight intravenous inoculations of 1 ml of infectious stock virus administered semiweekly. A test bleeding was made two weeks after the 8th inoculation followed immediately by one additional virus inoculation. The final bleeding was made two weeks after the booster dose was administered. Serum was removed from the clotted blood by centrifugation at 1500 rpm in a PR-2 International centrifuge, head #253. Antibody concentration was determined by standard tube neutralization tests in MDBK cells as described below. The serum was collected from the clotted blood and final neutralization titrations determined. The sera was stored at -20° C in screw cap tubes.

Anti-IBR virus serum was kindly furnished by Dr. Carbary, National Disease Laboratory, U.S.D.A., Ames, Iowa. This serum was tested for antibody content by the same procedure described below.

Serum Neutralization Tests: Neutralization tests were carried out in MDBK or HEK tissue culture tubes using standard techniques described for

herpes viruses (Scott, 1953). To determine antibody content of immune rabbit serum, 0.3 ml of 2-fold dilutions of serum were combined with an equal volume of virus diluted to give 100 $TCID_{50}$. The mixture was incubated at room temperature for 1-1-1/2 hours. A 0.2 ml aliquot of the mixture was then inoculated into each of two tissue culture tubes. All sera was inactivated at 56° C for 30 minutes prior to use. Virus dilutions without serum served as controls. All tests were read 2 days after the virus controls showed 25 percent CPE or greater. Neutralization results are expressed as the reciprocal of the highest dilution of serum causing complete inhibition of virus CPE.

Electron Microscopy: Primary canine thymus cells propagated in 3-ounce prescription bottles were inoculated and incubated with IBR virus in the identical manner as described previously (Hinz et al., 1968), except that cultures were harvested 6, 12, 24, 48 and 72 hours after infection.

For viewing by electron microscopy, the cells were washed twice with BSS and mechanically removed from the glass surface with a rubber policeman. The cells were pelletized by centrifugation in a PR-2 International centrifuge, head #253. The supernatant was decanted and the pelletized cells were cut into fragments and placed in 3.5% glutaraldehyde solution (Sabitini, 1963) buffered at pH 7.5-7.6. After 24 hours the cells were washed in Sorensen's buffer solution and fixed in 2% Osmium tetroxide for 1 hour (Millonig, 1961). The cells were dehydrated in increasing concentrations of ethanol followed by propylene oxide, propylene oxide-EPON and finally embedded in EPON 812 (Luft, 1961). Ultrathin sections were cut with diamond knives and placed on 300 mesh copper grids coated with stabilized Formvar. The grids were double stained

with uranyl acetate and lead citrate (Reynolds, 1963). All sections were examined with a Siemens Elmiskop 1A electron microscope.

Density Gradient: Linear potassium citrate gradients (density 1.04-1.40 g/cm^3) were prepared in 12 ml polypropylene centrifuge tubes using an apparatus similar to that described by Martin and Ames (1961). A 0.5 ml sample of concentrated virus was added to the top of each gradient. The tubes were centrifuged in an International preparative ultracentrifuge, model B-35, with a swinging basket head SB-206, at 33,000 rpm for 18 hours at 4° C.

Virus fractions were collected after centrifugation by two methods: (1) puncturing the tube at the bottom with an 18-gauge needle and collecting 8 drop fractions, (2) puncturing the tube through the side at a point slightly below the virus band and drawing the banded material into a 1 ml syringe using a 25-gauge needle. Four fractions of 0.3-0.5 ml were collected with the bevel of the needle facing upward. All fractions collected using both methods were examined for infectivity and bouyant density. In addition, fractions collected by method 2 were assayed by neutralization tests and plaque formation. Bouyant density determinations were computed for potassium citrate from weight measurements of a known volume of each fraction or from refractive index measurements of the potassium citrate in each fraction using a Bausch and Lomb precision refractometer. Each fraction was then compared with a standard curve based on refractive index measurements of known concentrations of potassium citrate.

Plaque Assay: Plaque formation of IBR and MDH-1 viruses were accomplished using a modified technique of Dulbecco and Vogt (1954). Cultures of MDBK cells were grown in 60 mm disposable plastic petri dishes (1 x 10^6 cells/plate) using EMEM-10. The cultures were washed 2 times with 5 ml of HBSS per plate and 1 ml dilutions of the desired virus were added. The cultures were incubated, with periodic agitation, under the same conditions indicated above. Following a 1-1-1/2 hour adsorption period the cells were washed with 5 ml HBSS per plate. A 5 ml overlay medium consisting of EMEM containing 2% inactivated fetal calf serum and 0.9% noble agar was added to each plate. The cultures were further incubated in a CO_2 atmosphere at 37° C for 5-6 days. Visualization of plaques was accomplished by adding a second layer of agar medium (2 ml) containing a 1:10,000 dilution of neutral red or by adding several drops of neutral red directly to the existing agar medium. Plaques were usually observed and counted the following day.

Complement Fixation Test: The Laboratory Branch complement fixation test was performed according to the method Casey (1965). Lyopholized guinea pig complement was purchased from Texas Biological Laboratories, Inc., Fort Worth, Texas. Hemolysin was purchased from Sylvania Company, Milburn, New Jersey. Red blood cells were obtained from sheep by jugular puncture. The blood was defibronated by shaking for 3 minutes in a flask containing glass beads. The beads were separated by passing the blood through several layers of gauze. An equal volume of Alsever's solution was added and the suspension stored at 4° C.

Antigen: The antigen preparation was accomplished by propagating MDH-1 virus in primary CK cells and concentrating by the same procedure as used in virus preparation for density gradient. Virus particles from P3-J cells were separated as described previously. The particles were concentrated as described above.

Anti-MDH-1 rabbit serum was prepared as described previously.

Fluorescent Antibody: MDBK cells grown in 5% CO_2 atmosphere on 20 x 20 mm coverslips were used. After decanting the growth medium, the cells were washed two times with HBSS and 1 ml of MDH virus titering 10^6 $TCID_{50}$/0.1 ml was added. The virus was allowed to adsorb for one hour at 37° C in 5% CO_2 and the coverslips were washed three times in HBSS. The infected cultures were then incubated for 3-4 days in petri dishes containing maintenance medium. The coverslips were removed when CPE was evident and washed two times with HBSS. The coverslips were placed in -20° C acetone for 4 hours. Cells not infected with MDH-1 virus were handled the same as infected cultures. Following fixation all coverslips were removed from the acetone and stored in a container at -20 C.

Acetone fixed MDBK cells on coverslips were thawed and 0.4 ml of test sera was added. The cells were incubated at 37° C in 5% CO_2 for 30 minutes, washed in 0.1 M phosphate buffered saline, pH 7.2 for 10 minutes, and 0.4 ml of flurescene isothiocyanate conjugated goat anti-human or sheep anti-rabbit serum was added. The cells were again incubated at 37° C for 40 minutes followed by a 10-minute wash in phosphate buffered saline. For mounting on 2" x 4" glass slides a fluid consisting of 9 parts glycerol and 1 part phosphate buffered saline was used.

The cells were examined for fluorescence at 450x magnification using a Leitz ortholux microscope. The microscope was fitted with a mercury vapor arc HBO 200-watt bulb.

RESULTS

Neutralization Studies: MDH-1 virus was tested with a number of anti-viral sera, sera obtained from normal and lymphosarcoma dogs, and pooled human gamma globulin. In addition to the negative serologic results reported previously (Mitchell et al., 1967), neutralization tests were found negative when MDH-1 was combined with antisera to canine herpes virus and herpes virus simiae as well as all other canine and human sera tested.

A positive neutralization reaction was found with anti-IBR serum. Cross neutralization tests were then performed with specific anti-MDH-1 serum and IBR virus. As seen in Table 1, a complete and reciprocal neutralization reaction exists between MDH-1 and IBR viruses.

Table 1. Cross neutralization reactions between MDH-1 and IBR viruses

Virus	Sera*	
	MDH-1	IBR
MDH-1	20	40
IBR	20	40

* Numbers represent reciprocal of the highest dilution of sera which completely neutralized 100 $TCID_{50}$ of virus.

Electron Microscopic Studies: Canine thymus cells infected with MDH-1 or IBR virus were examined by electron microscopy. Both viruses were found as naked particles in the nucleus within 12 hours after infection. Many of these particles were associated with the nuclear membrane of the cell within 24 hours following infection. MDH-1 viruses were often seen contained in an intranuclear viroplasmic-like mass (Figure 5), while the strain of IBR used exhibited only single naked particles randomly distributed throughout the nucleoplasm (Figure 6). The viruses acquired the nuclear membrane either by budding through the membrane or extruding from a damaged nucleus into the cytoplasm. Membrane associated particles were frequently found in the cytoplasm within 48 hours after infection. IBR viruses were found with single or double membranes at this time and were within the same size range as the MDH-1 virus. Mature particles were released when the integrity of the cell was destroyed by infection and were infectious when found in the supernatant fluid.

Tissue Culture Host Range: Cells in culture representing human, hamster, canine, mouse and bovine species, were inoculated with MDH-1 and IBR viruses and carried through five subpassages using a 7-10 day incubation period between subpassages. Results of these inoculations are summarized in Table 2.

When propagated in DEF, MDH-1 virus was not capable of completing its multiplication cycle in the 7-10 days between subpassages. The virus eventually adapted to the cell, but only after extended incubation of the infected culture. Conversely, IBR infected DEF cells produced infectious virus in each subpassage with an increase in virus titer with each passage.

Table 2. Infectivity titers of fifth subpassage material from infected cell cultures*

Cell Line	MDH-1	IBR
MDBK	7.25	7.50
DEF	Negative**	5.25
MDCK	Negative	Negative
L-929	Foci	Negative
BHK-21	Negative	Negative
HEK	5.5	Negative
Ch.L.	Negative	Negative
HeLa	Negative	Negative
Thymus	4.5	1.5

* Numbers represent reciprocal of log 10 tissue culture infectious dose - 50 percent/0.1 ml of virus preparation determined by assaying in MDBK cells. Cultures incubated 7-10 days between subpassage.

** MDH-1 infectious for DEF cells after extended cultivation of infected cells.

MDH-1 was found to propagate well in HEK cells with a maximum titer of 10^5 in the 5th subpassage. IBR virus produced CPE on initial inoculation of HEK cells, but CPE was not found following the 1st subpassage. Infectivity titrations of fluid from the 3rd and 5th subpassages demonstrated no infectious virus.

In studying the host ranges it was noted that MDBK cells were highly susceptible to MDH-1 virus. Upon inoculation of HEK cells with MDH-1 propagated through 2 passages in MDBK cells, no CPE or infectious virus was produced.

Mouse L cells were inoculated with MDH-1 stock virus, and the cells were observed daily for evidence of viral activity. On first subpassage representing the 9th day after inoculation, foci characteristic of transformed cells developed in the culture (Figure 7). Cells from these foci were isolated and have been carried in culture through 22 passages. Foci continued to develop through the 8th passage but then grew as confluent monolayers. The individual cells appeared larger and the cultures grew at a faster rate than L cells not infected with MDH-1. No infectious MDH-1 virus was recovered from these cells.

Electron microscope examination of the L cells indicated a greater number of C-type and A-type particles in the MDH-1 infected cells but no herpes-like particles were seen.

IBR infected cultures of mouse L cells did not produce foci, nor was infectious virus recovered from the cultures.

Density Gradient Assay: When MDH-1, IBR, or the P3-J virus preparation was layered on preformed gradients of potassium citrate and centrifuged for 18 hours, the viruses separated into several bands. The majority of the three viruses were found in thin distinct bands just below the midpoints of the tubes (Figures 9, 10 and 11). A second diffuse opalescent band was found slightly above the thin band and extended up the tube several millimeters. A cottony flocculent band associated with each of the three preparations was located below the thin virus band.

The densities of the various bands were determined and are summarized in Table 3, along with the density of the herpes-like particles separated from P3-J cells maintained in our laboratory. Data presented by Toplin and Schidlovsky (1966) concerning herpes-like particles recovered from

Table 3. Densities of MDH-1, IBR and the herpes particles isolated from cultured Burkitt lymphoma cells

Virus	Diffuse Band	Thin Band	Flocculent Band
		Density (g/cm^3)	
MDH-1	1.19-1.04	1.206	1.24-1.25
IBR	1.19-1.04	1.198	1.22-1.25
P3-J Preparation	N.D.	1.195	N.D.
EB-3 Preparation*	1.16-1.13	1.19-1.21	1.19-1.21

* Data from Toplin and Schidlovsky (1966).

the EB-3 line of Burkitt's lymphoma is included for comparison. A density of 1.20 was found for the thin band which contained the greatest concentration of all four viruses or virus-like particles.

The four gradient fractions of MDH-1 collected by puncturing the tube at the side, i.e., one fraction from the flocculent band, thin band, diffuse band and at the top of the tube, were also assayed using MDBK cells and concentrated by ultracentrifugation. These virus preparations were layered on potassium citrate gradients and centrifuged for 18 hours. The viruses separated into bands identical to that seen in Figure 9 for MDH-1.

Assay of Purified Virus from Gradients: Each of the four fractions of MDH-1, collected by puncturing the side of the tube, were studied by neutralization using specific antiserum and for plaque formation. The virus in each of the four fractions was grown in MDBK cells and virus preparations from these cells were titrated for infectivity. Specific MDH-1 and IBR antisera was combined with each of the four virus preparations and inoculated into MDBK cells. Neutralization for all were complete.

The plaque forming ability of the virus from each fraction was also examined. Each virus was adsorbed to MDBK cells in 60 mm petri dishes and overlaid with agar. Plaques formed in 5-6 days with all four virus preparations. The plaques were generally round with irregular edges, ranging in size from 0.5-1.5 mm in diameter.

Virus from a large and a small plaque were selected and grown separately in MDBK cells. Viruses prepared from the two plaque sizes were then inoculated onto MDBK cells in 60 mm petri dishes and overlaid with agar. Plaques which formed in 5-6 days ranged from 0.5-1.5 mm regardless of whether the virus originated from a large or a small plaque.

Complement Fixation Studies: Rabbit anti-MDH-1 serum was used as the source of specific antibodies for reaction in the LBCF test with the herpes-like particle recovered from the P3-J cells. MDH-1, IBR and the concentrated P3-J herpes-like particles extracted from the Burkitt cells were tested against specific MDH-1 and IBR antiserum and against serum from patients with Burkitt's lymphoma. The results of these reactions are presented in Table 4. A strong reaction occurred between the concentrated P3-J antigen and MDH-1 immune serum. A reaction also occurred when Burkitt patient serum was used. MDH-1 virus reacted strongly with specific immune serum and the Burkitt patient serum. All serum and antigen controls were negative.

Fluorescent Antibody Studies: A variety of sera were used in the indirect immunofluorescent antibody test against MDBK cells infected with MDH-1 virus. A fluorescent reaction was found in the nucleus of MDH-1 infected cells adsorbed with two of four sera from patients with Burkitt's

Table 4. Complement fixation reactions relating MDH-1, IBR and the herpes-like particle (P3-J) extracted from Burkitt lymphoma cells in culture*

Antigen 1:2	Sera MDH-1 1:16	IBR 1:8	Burkitt 1:8	Rabbit 1:16	Human 1:8
MDH-1	15	10	20	95	100
IBR	15	5	N.D.	95	N.D.
P3-J Part.	20	10	20	95	100
Hu. Ext.	95	N.D.	95	95	100
CK Ext.	100	N.D.	95	100	N.D.

* Numbers represent percent of hemolysis present in reaction tubes.

lymphoma (Table 5). A third serum caused a dull reaction with these cells. When sera from patients with infectious mononucleosis was used, a fluorescent reaction was observed in six of the ten sera tested. Heterophil

Table 5. Results of indirect immunofluorescent tests using sera from diseased and healthy humans against MDBK cells infected with MDH-1 virus

Sera from patients with:	Number Positive	Number Negative
Burkitt lymphoma	3*	1
Infectious mononucleosis	6	4
No disease	1	5

* One of these sera caused a weak fluorescent reaction.

antibody titers determined on these ten sera varied and were independent of the fluorescent reaction. The fluorescent reaction in the cells regarded as positive was characterized by spherical intranuclear structures varying in size. The fluorescence was generally weak but occurred in cells showing CPE.

GENERAL DISCUSSION

The frequent findings by electron microscopy of a herpes-like particle in the cultured cells of Burkitt's lymphoma (Epstein et al., 1965; Toshima et al., 1967) has presented the question of whether these particles represent the virus responsible for Burkitt's lymphoma, a passenger in the tumor tissue, or a contaminant infecting the cultured cells. These questions could not be answered until the herpes particle was isolated and grown as a productive virus, and its characteristics studied. An attempt was made to isolate the herpes-like virus present in the P3-J line of cultured Burkitt lymphoma cells. This attempt was based on the assumption that interaction with another virus was required.

Results of superinfecting the cultured P3-J cells with 18 different viruses (Table 8, Appendix) revealed these cells to have a wide susceptibility to viruses. Most human viruses which were found to propagate in the P3-J cells eventually destroyed the culture (Table 10, Appendix).

The cells inoculated with Reovirus type 3 were established as a chronically infected culture for over three months (Table 10, Appendix), during which time only reovirus 3 could be recovered. Bell et al. (1966) reported the isolation of Reovirus type 3 from tumor tissue of 10 patients with Burkitt's lymphoma and suggested that this agent was possibly the etiology. Evidence was subsequently presented (Bell and Ross, 1966) that this virus could be propagated as a persistent latent infection of human embryo fibroblast cells. The virus apparently did not have the ability to induce the herpes virus present in the P3-J cells to propagate in other cells. Rabson et al. (1966) found no evidence by electron microscopy of an increase in the numbers of herpes particles in the AL-1 line of Burkitt's

lymphoma cells superinfected with Reovirus type 3. These investigators were also unable to demonstrate an increase in the number of herpes particles in AL-1 cells superinfected with adenovirus types 7 and 12, vaccinia or SV-40.

A strain of adenovirus type 12 (GHS-399, Murphy) was established as a persistent latent infection in the P3-J cells. Viruses other than the superinfecting virus were not demonstrated in the host cells used in these studies. In addition, adenovirus 7 and SV-40 were established as carrier cultures for 3-4 weeks without cell destruction after which time infectious virus could not be demonstrated. The failure of these and other human and monkey viruses (Table 11, Appendix), which propagated in P3-J cells, to induce the herpes virus into a productive state, indicates that a factor or factors other than an active virus infection in the P3-J cells was required.

One P3-J culture inoculated with Moloney sarcoma virus (MSV) presented interesting results different from the cultures inoculated with the other viruses. MSV was added to the culture of P3-J cells on the same day that seven other candidate helper viruses were added to cultures of P3-J cells. Growth fluid in each of these cultures was changed every 3-4 days. The growth fluid from each culture was added to culture tubes containing HEK, WI-38, CT or CK cells. Examination of cells inoculated with supernatant fluid from P3-J cultures superinfected 10 days previously with MSV revealed CPE in the CT cells. The type of CPE and lack of inhibition of CPE by antibodies specific for PPLO suggested that this agent was a virus. Results of mouse inoculations and cultured cells derived from mice, and the fact that anti-MSV serum failed to inhibit the growth of virus, indicated that the virus (MDH-1) was not related to MSV.

It seems likely that MSV played an important role in the recovery of MDH-1 from the P3-J cells, despite the failure to repeat the recovery of a similar virus from other P3-J cells superinfected with MSV. The exact mechanism by which MSV induced the release of this infectious virus from the P3-J cells has not been elucidated. There was no evidence of a necrotizing virus in any of the control cultures carried with the P3-J culture inoculated with MSV. The controls included: CT cells inoculated with the original stock supply of MSV; the noninoculated CT cells; CT cells inoculated with supernatant fluid from two cultures of P3-J cells not superinfected with virus; and supernatant from seven cultures of P3-J cells superinfected with other viruses. Media used for all the P3-J cells was from a common source. Media used for all CT cells also came from a common source. It appears very unlikely that MDH-1 could have originated from any source other than the P3-J cells following an interaction with MSV.

The question of the origin of MDH-1 virus could not be answered until the virus was characterized and a relationship shown with the virus in the P3-J cells or with a virus from some other source. MDH-1 virus was examined by biological and physical means in an effort to establish this relationship.

Ultrastructural examination has disclosed that morphologically, MDH-1 virus observed in infected CT cultures in all respects was a typical herpes agent (Figures 1 through 20, Publication 2). Electron dense particles occurred in the cytoplasm and nucleus before there was evidence of incomplete virus formation. Shipkey et al. (1967) found similar electron dense particles, resembling virus nucleoids and measuring 30-60 mμ in diameter, only in the nucleus in herpes simplex infections. The importance

of MDH-1 virus induced dense particles being consistently present within the cytoplasm, migrating and becoming associated with the outer nuclear membrane (Figures 1 and 2, Publication 2) is not known.

Cell cultures infected with herpes simplex virus showed convolutions of nuclear membrane (Falke et al., 1959), reduplication of the membrane (Morgan et al., 1959), and thickening of the nuclear membrane (Watson et al., 1964). These same alterations were seen in CT cells infected with MDH-1 virus.

When MDH-1 virus appeared to form at random throughout the nucleus, degeneration of the intact cell was not as marked (Figures 7 and 11, Publication 2). Conversely, when naked virus particles were seen contained in the very dense, sharply delineated viroplasmic-like mass (Figures 6 and 8-10, Publication 2), the nucleus showed a marked increase in the chromatin margination and seemed to be devoid of nucleoplasmic substance (Figure 6, Publication 2). The reasons for, or significance of, these two phenomena in naked virus particle formation is not known; however, involvement of a different cell type in the CT culture might be one possibility. Another might be that the virus contained two population segments which had different developmental patterns. In either event the formation of naked virus particles was preceded by 35-40 mμ electron dense particles which appeared in both the cytoplasm and the nucleus. It was assumed that most of these dense intranuclear particles were formed in the nucleus and could very easily represent viral DNA.

The naked virus particles began as a single or double hexagonal shell and acquired its nucleoid at a later time, possibly from the inner membrane of the double shelled particles. Similar particle formation

was described by Becker et al. (1965) in cells infected with herpes zooster and cytamegalovirus and by Epstein et al. (1962) for herpes simplex virus.

It was common to see single shelled particles attached to the inner nuclear membrane and budding through and into the cytoplasm in those cells where naked virus particles were randomly distributed through the nucleoplasm. Maturation of MDH-1 virus occurred by being discharged through a damaged nucleus into the cytoplasm. The budding viruses were, in general, more pleomorphic and larger than those discharged through a damaged nuclear membrane. Thickening of the inner nuclear membrane did not seem to be associated with naked particle attachment, which was consistent with the findings of Falke et al. (1959).

The formation of double and triple membrane associated viruses took place in direct association with nuclear membrane material. When this occurred in the cytoplasm, it was a function of the reduplication and/or proliferation of the nuclear membrane which had dissociated from the main body of the nucleus and became localized in the cytoplasm. The virus was released from the cell when the integrity of the cell was destroyed by virus infection. The size of the mature particles was 110-160 mμ in diameter, which was within the size range of other herpes viruses (Armstrong et al., 1961; Becker et al., 1965; Wildey, 1960).

A similarity occurred between MDH-1 virus and other herpes viruses in regard to the visible cytopathogenic effects in tissue culture (Figures 1 and 2). Cowdry type A intranuclear inclusions were frequently found in cultures of CT cells after routine fixing and staining procedures (Figure 3). These inclusions resembled intranuclear structures described for herpes simplex virus (Scott et al., 1953), infectious bovine rhinotracheitis virus (Armstrong et al., 1961) and others.

The mature virus was enveloped with membranes susceptible to degradation by ether, which was in agreement with Kaplan and Vatter (1959) for herpes simplex and pseudorabies viruses.

The morphologic appearance, size and structural development of MDH-1, the formation of type A intranuclear inclusions and the ether sensitivity clearly established this virus as a member of the herpes group.

An attempt was made to demonstrate a serologic relationship between MDH-1 and a previously identified virus. Neutralization tests, using specific antiviral sera, revealed MDH-1 virus to have a strong reciprocal, serologic relationship to infectious bovine rhinotracheitis virus, suggesting that these two viruses were either similar or shared a common antigenic component.

When MDH-1 virus was compared to the ultrastructural characteristics of IBR virus, using the electron microscope, a similarity was again found. Both viruses were in the same size range (110-160 mμ) and had similar developmental stages. In our laboratory, the naked IBR particles were not found replicating in an intranuclear viroplasmic-like mass when using the L.A. strain as was found with the MDH-1 virus (Figure 5). Gratzek et al. (1966) have reported structures similar to the viroplasmic mass in the nucleus of primary bovine testicular cells infected with the ISU-1 strain of IBR virus.

Since MDH-1 and IBR viruses appear the same serologically, one might expect that their tissue culture host range would be the same also, since this is a criterion for virus classification (Lowoff et al., 1962). Although most of the cell lines tested had the same susceptibility spectrum, three lines HEK, CEF and L-929 cells responded differently to the two viruses.

MDH-1 propagated well in HEK cells but IBR did not have a complete infectious cycle in these cells. Conversely, the canine embryo cells supported IBR virus replication well, while MDH-1 replicated only after extended cultivation between subpassages. With a 7-10 day incubation period no MDH-1 was found in the fifth subpassage. These findings presented clear evidence that the two viruses possessed some differences in their genetic markers.

The study of the interaction of MDH-1 virus and the L-929 cells is indeed of interest, since these cells have been found to contain C and A type virus-like particles when viewed by electron microscopy (Dales and Howatson, 1961; Kindig and Kirsten, 1967). A relationship between C type RNA viruses and the herpes viruses found in the cultured cells of Burkitt's lymphoma was suggested by results presented by Fink and Cowles (1968) using immune serum prepared against C type particles concentrated from the plasma of leukemic inddviduals. A positive immunofluorescent reaction was found when this serum was reacted against the cultured Burkitt lymphoma cells containing herpes-like particles. A serologic reaction was also found when this serum was reacted against herpes particles extracted from the cultured cells and tested by the gel double diffusion technique.

The development of foci following inoculation of L-929 cells with MDH-1 presented more evidence of a relationship between these herpes viruses and the RNA viruses. These cells were transformed prior to inoculation of MDH-1 virus so that the expression of an oncogenic characteristic by MDH-1 virus could not be evaluated by the usual methods. Although

it was shown that hamster cells have been doubly transformed by two different oncogenic viruses (Takemoto and Habel, 1966), the fact that the foci persisted for only eight passages suggested that the MDH-1 in some way "activated" the L-929 cells to express a transformation characteristic which it already possessed.

There was no evidence of foci formation when IBR virus was inoculated into the L-929 cells, but herpes simplex caused a localized degenerative foci resulting in the formation of multinucleated giant cells (Garabedian and Scott, 1967).

The host range findings, the strong serological relationship and the similarities seen by the electron microscope indicated that MDH-1 virus was a strain of IBR virus, but probably different than the L.A. strain of IBR with which it was compared.

An attempt was then made, using physical and serological techniques, to show a relationship between MDH-1 and the other herpes-like particles found in the cultured Burkitt lymphoma cells. One definite similarity between MDH-1, IBR, the herpes-like particle recovered from the P3-J cells and a nondigested herpes-like particle recovered from the EB-3 line by Toplin and Schidlovsky (1966) was in the bouyant density of these agents. The greatest concentration of these four agents was found at a density of approximately 1.20. Viruses from the four sources were also found in a lighter band that extended in some cases to the sample zone. Positioning of the cottony flocculent band of the EB-3 preparation as reported by Toplin and Schidlovsky (1966) was found with the virus band at 1.20, while the MDH-1, IBR and P3-J flocculent bands were found below the virus band.

The MDH-1 recovered from the various bands probably represented a population of particles with heterologous densities, since virus was found in all fractions, was neutralized by specific antisera and had identical plaque characteristics. In addition, virus from each of the four bands when separated by density gradient centrifugation, after being propagated in MDBK cells, again banded the same as the original virus.

A comparison of the ultrastructural characteristics of MDH-1 and the herpes-like particles found in the cultured Burkitt cells (Toshima et al., 1967; Epstein et al., 1964) revealed a striking similarity in size, structure and developmental stages.

Based on the results obtained from the density gradient and electron microscope studies, it seems likely that MDH-1 virus is similar to the virus found in the Burkitt lymphoma cells. This hypothesis was supported by the findings of a complement fixation reaction between Burkitt patient serum, MDH-1 virus and the virus particles extracted from the P3-J cells. Gerber and Birch (1967) found complement fixing antibodies in sera from patients with Burkitt's lymphoma against viral antigens extracted from P3-J cells.

MDH-1 infected MDBK cells, when combined with sera from patients with Burkitt's lymphoma, were found to produce a positive immunofluorescent reaction in the nucleus. Henle and Henle (1966a) found a brilliant membrane staining in a small number of cells in cultures derived from patients with Burkitt's lymphoma and other diseases but were unable to demonstrate a positive fluorescent reaction in these cells when combined with a variety of specific herpes virus antisera, including anti-IBR serum (Henle and Henle, 1966b). The reason for the difference in our findings

and those of others (Henle and Henle, 1966b) is not known but could be that the antibodies demonstrated by Henle and Henle (1966b) were against nonstructural antigens similar to the tumor antigens found in adenovirus transformed hamster cells (Hoggan et al., 1965), while the MDH-1 antibodies were against structural antigens. Sera from Burkitt patients, when combined with MDH-1 infected MDBK cells, caused a fluorescent reaction which was identical in apperance to the reaction found when specific anti-MDH-1 serum was used.

A relationship between antibodies associated with infectious mononucleosis and the herpes-like particles in the cultured Burkitt cells has been suggested from positive immunofluorescent and complement fixation reactions (Henle et al., 1968; Gerber, 1968). Attempts to show a relationship between MDH-1 and antibodies in the sera from patients with infectious mononucleosis have resulted in a positive nuclear immunofluorescent reaction in 6 of 10 sera tested against MDH-1 infected MDBK cells.

The fact that MDH-1 was a productive virus and the herpes viruses in the cultured Burkitt cells are nonproductive suggests that MDH-1 possesses some genetic characteristics that are not found in the herpes viruses in the Burkitt cells in culture. The origin of this characteristic has not been established, but it probably resulted from the interaction with MSV. It seems unlikely that the genetic material of MSV is incorporated in the MDH-1 virus since none of the characteristics of MDH-1 were shown to be related to MSV. Two possibilities remain: (1) the herpes particle or the P3-J cells were affected by the interaction with MSV allowing a new population segment to be produced, and

(2) an interaction between MSV and a contaminating virus such as IBR occurred and that the IBR virus acted as a helper to the herpes particle in the P3-J cells. It should be mentioned that at the time of recovery of MDH-1, IBR virus was not present as a stock virus in the laboratory. There is a possibility that many of the herpes viruses commonly found in the cultured cells of Burkitt's lymphoma have arisen from the fetal calf sera which supplemented the media used to feed the cultured Burkitt cells. This suggestion is based on the similarity of MDH-1, IBR, the P3-J particle, the EB-3 particle, and the finding of herpes-like particles in cultured leukocytes from normal human donors (Gerber and Monroe, 1968).

As noted previously, the CT passage of MDH-1 virus propagated well in HEK cells but MDH-1 failed to propagate in HEK cells after two passages in MDBK. Assuming MDH-1 and IBR act the same, an IBR virus originating in bovine cells which gained access to human cells would be capable of initiating an infectious cycle in human cells but would not release a productive virus.

The loss of ability to propagate was not the result of acquiring a bovine associated coat, as evidence of an infection cycle was found when the bovine propagated MDH-1 was added to HEK cells. It was also unlikely that there would be a change in the genetic structure of the virus after only two passages in bovine cells.

The biological and physical characteristics described indicate that MDH-1 and IBR are similar viruses. From the preceding information it is suggested that these viruses are the same as those found in the Burkitt lymphoma cells in culture.

SUMMARY

SUMMARY

Cultured Burkitt lymphoma cells (P3-J) were superinfected with a variety of viruses derived from human, canine, monkey and mouse origin in an attempt to recover a latent or passenger virus present in the P3-J cells. Supernatant fluid from the infected cultures was selected at 3-4 day intervals and added to cultures of canine thymus, canine kidney, and human diploid (WI-38) cells. The inoculated cells were examined for evidence of a virus with properties different from that of the superinfecting virus by studying the host range, neutralization by homologous antiserum and other biological characteristics.

A virus, designated MDH-1, was recovered from a P3-J culture superinfected with Moloney sarcoma virus. An attempt was made to determine if the agent recovered was MSV. The virus was not neutralized by antiserum specific to Moloney sarcoma virus. It did not produce tumors in the newborn mice when inoculated intramuscularly, and cytopathogenic effects and/or transformation were not observed in cultures of mouse embryo fibroblasts.

The virus was originally isolated in cultures of primary canine thymus cells. CPE was evident in the thymus cells 7-10 days after addition of culture fluid from Moloney sarcoma virus infected P3-J cells. Infectivity titers of MDH-1 virus in the thymus cells were $10^{4.5}$/0.1 ml. Subpassage of the virus to human embryonic kidney cells also resulted in production of CPE and infectious virus. Infectivity titers in HEK cells were $10^{5.0}$/0.1 ml.

Virus titering $10^{4.5}$/0.1 ml was completely inactivated after exposure to 56° C for 30 minutes. The virus was also completely inactivated when treated for one hour with an equal volume of ether but was not inhibited by 10 μg/ml of tetracycline or 5 mg/ml of amphoterison B (Kanamycin).

Cowdry type A intranuclear inclusions were commonly found in MDH-1 infected canine thymus cells when stained by the May-Grunwald-Giemsa technique and examined by the light microscope. Detailed ultrastructural studies, using the electron microscope, of the developmental stages at different intervals after infection of canine thymus cells revealed the virus to have typical morphologic and developmental patterns common to herpes viruses. The size of the naked (immature) particle was 70-80 mμ in diameter, while the membrane associated virus was 110-160 mμ in diameter. Both naked and mature particles were in the size range of herpes simplex virus. Electron dense particles averaging 35 mμ in diameter formed in the cytoplasm in a juxtanuclear membrane position. Similar particles appeared in the nucleus 6-8 hours after infection and concurrently with naked virus formation. The naked virus was formed as a dense, sharply limited viroplasmic-like mass in the nucleus or as single particles randomly distributed in the nucleus. The mature virus formation resulted by a process of budding through the nuclear membrane. Virus was released from the cell by budding through the cytoplasmic membrane or released from the cytoplasm following cell destruction. The biological and physical characteristics along with ultrastructure suggest that the MDH-1 is a herpes virus.

MDH-1 virus was demonstrated to have a strong and reciprocal serologic relationship to infectious bovine rhinotracheitis virus. MDH-1 virus had no serologic relationship to any other human or canine herpes virus or to a variety of other human viruses, including adenoviruses, reoviruses and poxviruses.

Comparison of the ultrastructural characteristics of MDH-1 to IBR virus, by electron microscopy, revealed both viruses to be in the same size range and to have many of the same structural characteristics. Immature IBR virus was found as single particles randomly distributed throughout the nucleus but not as dense viroplasmic-like masses.

Comparison of the tissue culture host range of MDH-1 and IBR viruses disclosed differences in three cell lines: HEK, canine embryo (CE) and Earle's L-929 cells. MDH-1 virus was shown to propagate in HEK cells. CE cells supported the growth of IBR virus but MDH-1 virus could be propagated only after extended cultivation of the CE cells. The Earle's L-929 cells responded to MDH-1 virus with foci formation after 9 days in culture, while IBR produced no change in these cells.

The similarity in the serologic reactions between MDH-1 and IBR viruses and the developmental structure coupled with the lack of similarity of host range suggest that these viruses represent two strains of the same virus.

Examination of MDH-1 and IBR viruses after centrifugation in a potassium citrate gradient reveal the virus to have a density of 1.20. Virus particles from P3-J cells were extracted, concentrated and layered on a potassium citrate gradient. Examination of the density of these particles after centrifugation disclosed the particles to have a density identical to the MDH-1 virus.

The size of both naked and mature MDH-1 virus was in the same range as reported by others for the herpes particles commonly found in the cultured Burkitt lymphoma cells. The release of the herpes-like particle from the nucleus of Burkitt cells was occasionally by budding through the nuclear membrane but more frequently by being extruded into the cytoplasm through damaged nuclear membranes.

MDH-1 virus was used as an antigen to prepare specific anti-MDH-1 serum. This serum produced a positive complement fixation reaction when tested against MDH-1 virus. A positive complement fixation reaction was also found when concentrated herpes-like particles, from the P3-J cells, were used as an antigen against the specific anti-MDH-1 serum.

MDH-1 was not neutralized when combined with sera from patients with Burkitt's lymphoma. When this sera was adsorbed to bovine cells infected with MDH-1 virus, for use in an indirect immunofluorescent test, a positive fluorescent raction was observed with 3 of 4 sera tested. When MDH-1 virus infected bovine cells were adsorbed with sera from patients with infectious mononucleosis, for use in an indirect immunofluorescent test, a positive reaction was again found.

The similarities in density, size and appearance, and the serological reactions between MDH-1 virus and the herpes particles in the cultured cells of Burkitt's lymphoma, suggest that MDH-1 virus and herpes particles are the same or closely related.

FIGURES

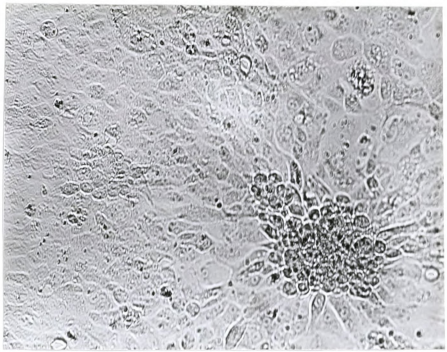

Figure 1. Early stage of cytopathic effects in bovine kidney cells infected with MDH-1 virus. X 150.

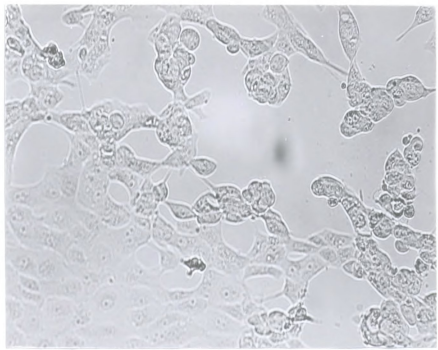

Figure 2. A late stage of cytopathic effects in bovine kidney cells infected with MDH-1 virus. X 165.

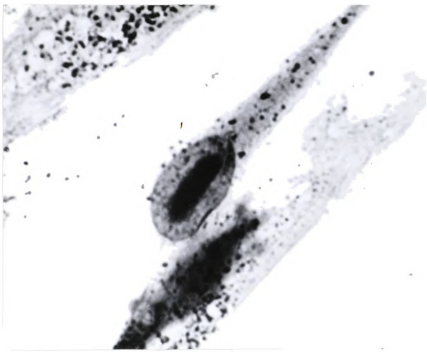

Figure 3. May-Grunwald-Giemsa stained MDH-1 infected canine thymus cells containing Cowdry type A intranuclear inclusions. X 1850.

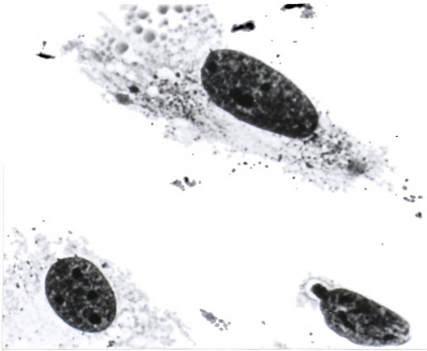

Figure 4. Normal canine thymus cells stained with May-Grunwald-Giemsa reagent. X 1850.

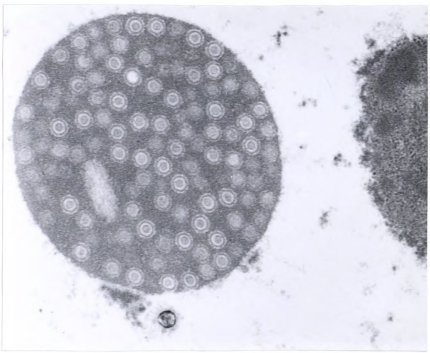

Figure 5. Canine thymus cells with single-shelled MDH-1 virus particles replicating in the nucleus in a dense viroplasmic mass, 18 hours after infection. X 18,000.

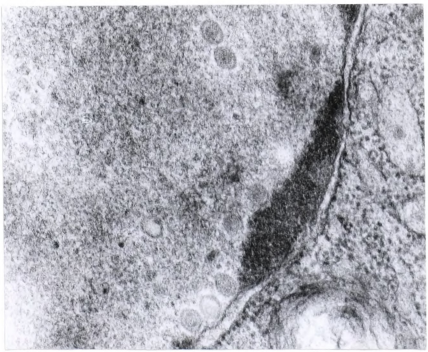

Figure 6. Randomly distributed, single-shelled IBR virus particles replicating in the nucleus. X 90,000.

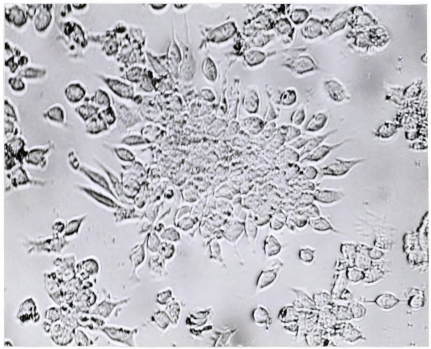

Figure 7. Mouse L-929 cells containing foci after infection with MDH-1 virus. X 320.

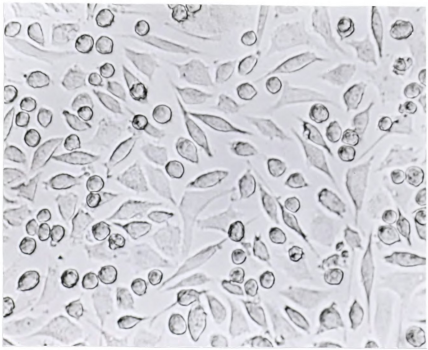

Figure 8. Normal L-929 cells. X 350.

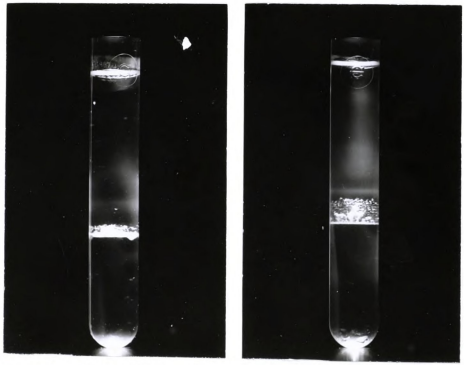

Figure 9. Gradient of potassium citrate containing MDH-1 virus centrifuged 18 hours at 33,000 rpm.

Figure 10. Gradient of potassium citrate containing IBR virus centrifuged 18 hours at 33,000 rpm.

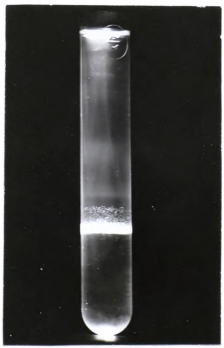

Figure 11. Gradient of potassium citrate containing herpes particles extracted from P3-J cells and centrifuged 18 hours at 33,000 rpm.

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APPENDIX

Table 6. Cell cultures

Name and Designation	Tissue of Origin	Animal of Origin	Continuous / Discontinuous
Burkitt (P3-J)	Lymphoma	Human	C
HEK	Kidney	Human	D
WI-38 (diploid)	Lung	Human	D
Hu. A.	Amnion	Human	D
HeLa	Carcinoma	Human	C
Chang Liver	Liver	Human	C
CEF	Embryo	Canine (Beagle)	D
CT	Thymus	Canine (Beagle)	D
CK	Kidney	Canine (Beagle)	D
MDCK	Kidney	Canine (Spaniel)	C
MDBK	Kidney	Bovine	C
BHK-21	Kidney	Hamster (Syrian)	C
NCTC clone 929 (Strain L)	Connective	Mouse (C3H)	C
MEF	Embryo	Mouse	D
RMK	Kidney	Monkey (rhesus)	D
AGMK	Kidney	Monkey (green)	D

MBA - Microbiological Associates, Inc., Bethesda, Maryland.

Table 7. Source of cells and type of media used to cultivate cells

Culture and Designation	Source	Media and % Serum		Method of Dispersing for Transplantation
		Growth	Maintenance	
P3-J	Pfizer	McCoy's-20	McCoy's-5	Pipette
HEK	MBA	HBME-10	EBME-5	Trypsin
WI-38	MBA	HBME-10	EBME-5	Trypsin
Hu. A.	MDH	HBME-10	EBME-5	Trypsin
HeLa	ATCC	HBME-10	EBME-5	Trypsin
Chang Liver	ATCC	HMEM-10	EMEM-5	Trypsin
CEF	MSU	HMEM-10	EMEM-5	Trypsin
CT	MSU	H199-20	E199-5	Trypsin
CK	MSU	HBME-10	EBME-5	Trypsin
MDCK	ATCC	HMEM-10	EMEM-5	Versene-trypsin
MDBK	ATCC	HMEM-10	EMEM-5	Versene-trypsin
BHK-21	ATCC	HMEM-10	EMEM-5	Trypsin
NCTC 929(L)	ATCC	HMEM-10	EMEM-5	Trypsin
MEF	MDH	HBME-10	EBME-5	Trypsin
RMK	MBA	H199-5	E199-1	Trypsin
AGMK	MBA	H199-10	E199-1	Trypsin

MBA - Microbiological Associates, Inc., Bethesda, Maryland.

MDH - Michigan Department of Health, Lansing, Michigan.

ATCC - American Type Culture Collection, Rockville, Maryland.

Table 8. Strains of viruses used to superinfect P3-J cells

Virus	Strain Designation	Host Cell	Infectivity Titer*
Reovirus-1	#12610 (ECHO-10)	RMK	5.00
Reovirus-2	Jones	RMK	5.00
Reovirus-3	Abeny	RMK	5.00
Parainfluenza-1	HA-2 Strain C35	HEK	3.50
Parainfluenza-3	HA-1 Strain C243	HEK	4.50
Parainfluenza-3	Murphy E-869 (wild)	HEK	3.50
Adenovirus-4	Dutoit	HEK	7.00
Adenovirus-5	Adenoid 75 (NIH)	HEK	5.50
Adenovirus-7a	S-1058 (NIH)	HEK	6.50
Adenovirus-9	Hill	HEK	6.00
Adenovirus-12	Murphy GHS-399 (wild)	HEK	6.00
Vaccinia	MDH	HEK	5.50
Herpes simplex	MacIntyre YS-12	HEK	4.25
Canine herpes virus	F-205-V	CK	4.50
Infectious canine hepatitis	Cornell-1	CK	5.00
Murine sarcoma	Moloney	MEF	N.D.
Murine leukemia	Moloney	MEF	N.D.
Simian virus-40	MDH	AGMK	3.00

* Numbers represent the reciprocal of the highest dilution of virus showing cytopathogenic effects. Calculations based on results in three tubes per dilution, according to Reed and Muench (1938).

Table 9. Susceptibility of CT, CK, HEK and WI-38 cells to selected viruses

Virus	Infectivity Titers*			
	CT	CK	HEK	WI-38
Reovirus-1	1.25	3.50	6.75	3.50
Reovirus-2	5.50	2.25	5.50	4.00
Reovirus-3	3.24	1.50	6.75	3.75
Parainfluenza-1	None	None	None	None
Parainfluenza-3 (HA-1)	N.D.	N.D.	4.50	N.D.
Parainfluenza-3 (Murphy)	None	None	3.50	None
Adenovirus-4	None	None	7.00	2.50
Adenovirus-5	None	None	5.50	3.50
Adenovirus-7a	None	None	6.50	3.00
Adenovirus-9	None	None	6.00	2.75
Adenovirus-12 (GHS-399)	None	None	4.50	2.50
Vaccinia	4.50	7.50	5.50	3.50
Herpes simplex	None	None	4.25	1.50
Canine herpes	4.00	4.50	None	None
Infectious canine hepatitis	5.50	5.00	None	None
Murine sarcoma	None	None	None	None
Murine leukemia	None	None	None	None
Simian virus-40	None	None	None	None

* Numbers represent the reciprocal of the highest dilution of virus in which CPE was evident. Infectivity titers determined on supernatant fluid from virus infected cell (CT, CK, HEK or WI-38) by propagation in susceptible cell for each virus.

Table 10. Viruses inoculated into P3-J cultures and then propagated in the appropriate susceptible cell for each virus

Virus	Virus Production (days)	Culture Condition (days)	Infectivity Titer*	Neutralization of virus
Reovirus-1	17	17 (destroyed)	4.50	Complete
Reovirus-2	17	17 (destroyed)	4.50	Complete
Reovirus-3	105	105	6.50	Complete
Parainfluenza-1	None	38	None	N.D.
Parainfluenza-3	14	14 (destroyed)	3.50	Complete
E-869	None	46	None	N.D.
Adenovirus-4	None	78	None	N.D.
Adenovirus-5	18	18 (destroyed)	6.50	Complete
Adenovirus-7a	17	36	5.50	Complete
Adenovirus-9	18	18 (destroyed)	3.50	Complete
Adenovirus-12	105	105	4.00	Complete
Vaccinia	17	17 (destroyed)	2.25	Complete
Herpes simplex	16	16 (destroyed)	5.00	Complete
Canine herpes	None	78	None	N.D.
Infectious canine hepatitis	None	46	None	N.D.
Murine sarcoma	10	53	6.0	None
Murine leukemia	None	68	None	N.D.
Simian virus-40	26	102	2.50	Complete

* Numbers represent the reciprocal of the highest dilution at which CPE was evident. Infectivity titers determined on supernatant fluid from virus infected P3-J cells by propagating in susceptible cell for each virus.

Table 11. Susceptibility of CT, CK, HEK and WI-38 cells to viruses propagated in P3-J cells

Virus	Host Cells*			
	CT	CK	HEK	WI-38
Reovirus-1	+	+	+	+
Reovirus-2	+	+	+	+
Reovirus-3	+	+	+	+
Parainfluenza-1	-	-	-	-
Parainfluenza-3	N.D.	N.D.	+	N.D.
Parainfluenza-3 (E-869)	-	-	-	-
Adenovirus-4	-	-	-	+
Adenovirus-5	-	-	+	+
ADenovirus-7a	-	-	+	+
Adenovirus-9	-	-	+	+
Adenovirus-12 (GHS-399)	-	-	+	+
Vaccinia	+	+	+	+
Herpes simplex	-	-	+	+
Canine herpes	-	-	-	-
Infectious canine hepatitis	-	-	-	-
Murine sarcoma	-**	-	-	-
Murine leukemia	-	-	-	-
Simian virus-40	-	-	-	-

* The + sign indicates that the virus propagated in these cells.

** A virus propagated in CT cells was not the murine sarcoma virus but a herpes virus (see text).

Table 12. Effects in suckling mice of cell free supernatant fluid from P3-J cells superinfected with various viruses

Virus	Susceptibility of newborn mice		Neutralization of virus by specific serum
	Propagated in P3-J	Not propagated in P3-J	
Reovirus-1	None	None	N.D.
Reovirus-2	None	None	N.D.
Reovirus-3	+	+	Complete
Parainfluenza-1	None	None	N.D.
Parainfluenza-3 (E-869)	None	None	N.D.
Adenovirus-4	None	None	N.D.
Adenovirus-5	None	None	N.D.
Adenovirus-7a	None	None	N.D.
Adenovirus-9	None	None	N.D.
Adenovirus-12 (GHS-399)	None	None	N.D.
Vaccinia	+	+	Complete
Herpes simplex	+	+	Complete
Canine herpes	None	None	N.D.
Infectious canine hepatitis	None	None	N.D.
Murine sarcoma	+	None	N.D.
Murine leukemia	+	None	N.D.

* The + sign indicates that the virus in question propagated in newborn mice.

Table 13. Anti-viral sera

Antiserum prepared against	Animal Source	Neutralizing antibody titer vs. 100 $TCID_{50}$ of MDH-1 virus	Obtained from
Reovirus-1	Rabbit	1:800	MDH
Reovirus-2	Rabbit	1:800	MDH
Reovirus-3	Rabbit	1:800	MDH
Parainfluenza-1	Rabbit	1:80	MDH
Parainfluenza-3	Rabbit	1:400	MDH
Adenovirus-4	Rabbit	1:200	MDH
Adenovirus-5	Rabbit	1:100	MDH
Adenovirus-7a	Rabbit	1:400	MDH
Adenovirus-12	Rabbit	1:80	MDH
Adenovirus-18	Rabbit	1:800	MDH
Vaccinia	Rabbit	1:400	MDH
Herpes simplex (1)	Rabbit	1:640	CDC
Herpes simplex (2)	Rabbit	1:800	MBA
Canine herpes	Canine	1:100	Cornell U.
Infectious canine hepatitis	Canine	1:1280	Cornell U.
Murine sarcoma	Mouse	not titered	NCI
Murine leukemia	Mouse	not titered	NCI
Herpes simian (1)	Monkey	1:64	MDH
Herpes simian (2)	Horse	1:32	Upjohn Co.
MDH-1	Rabbit	1:40	Author

MDH - Michigan Department of Health, Lansing, Michigan.
CDC - Communicable Disease Center, Atlanta, Georgia.
MBA - Microbiological Associates, Inc., Bethesda, Maryland.

Table 14. Sera tested for detectable neutralizing antibodies against 100 $TCID_{50}$ of MDH-1 virus

Serum	Number Tested	Number Positive	Disease	Dilution Tested
Human	24	2	Leukemia	1:10
Human	26	0	Lymphoma	1:10
Human	12	1	Infectious mononucleosis	1:10
Human	5	0	Healthy	1:5
Canine	16	0	Lymphosarcoma	1:10
Canine	5	0	Healthy	1:10
Bovine	6	5	Healthy	1:10