STUDIES ON A PERSISTENT INFECTION OF BABY HAMSTER KIDNEY-21 CELLS WITH MUMPS VIRUS

by

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A THESIS

Presented to the Department of Microbiology and Immunology
and the Graduate Division
of the University of Oregon Health Sciences Center
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
February 1977

APPROVED:

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(Chairman, Graduate Council)

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INTRODUCTION

I. Statement of the Problem

Mumps is a paramyxovirus that causes epidemic parotitis, a common contagious disease of children and young adults characterized by inflammation of the salivary glands, especially the parotid. Frequent complications include orchitis in young men, and meningoencephalitis. The agemt has been implicated in clinical disorders such as viral arthritis, diabetes, primary endocardial fibroelastosis, and a number of other organ involvements.

The virus morphologically resembles other paramyxoviruses. It is a pleomorphic virion 100-300 nm in diameter, with a helical nucleocapsid 18 nm in diameter (S antigen). The intact virus particle has a lipid envelope about 100 Å thick which is covered with the spiked hemagglutinin (V antigen). The virus is sensitive to ether and chloroform, and infectivity is destroyed at 55 C for 20 min. Treatment with formalin destroys infectivity but not the hemagglutinin. The hemagglutinin agglutinates chicken, guniea pig, and human "O" red blood cells at room temperature or 4 C. The hemagglutinin is eluted from red blood cells at 37 C. The single stranded RNA has a molecular weight of approximately 7×10^6 daltons. The nucleocapsid and hemagglutinin are synthesized in the cytoplasm. The site of RNA synthesis is unknown, but the data suggest a cytoplasmic location. An RNA-dependent

RNA polymerase is associated with the mumps virion. The general properties of the reaction appear to be similar to those described for other paramyxoviruses. Mumps virus is composed of six polypeptides, ranging in size from 40,000 to 66,000 daltons, two of which are glycoproteins. The heavier glycoprotein contains both the neuraminidase and hemagglutinating activity.

All strains contain a common complement-fixing viral (V) and soluble (S) antigen. No strain differences exist by neutralization with mumps virus antiserum. A delayed hypersensitivity state is induced by active infection and can be observed with a skin test using live or inactivated virus.

Persistent infections with mumps virus have been described in L and Lung-To cell cultures, and in human conjunctival cells. The significance of the study of *in vitro* persistent infections as model systems for *in vivo* persistent infections and disease processes has been recognized for some time. The need for model systems for mumps virus is apparent due to its possible involvement in the chronic disease processes mentioned above. In addition, the use of attenuated strains of mumps virus in vaccine preparations raises the possibility that the vaccine virus may lead to a persistent infection in the host because of the decreased virulence. The purpose of this research was to study a persistant infection of mumps

virus in BHK-21 cells. The primary goals of the research were to characterize the cellular and viral parameters of the system and to study the properties responsible for the maintenance of the virus-cell interaction of this persistent infection. Increased understanding of this persistence may provide clues relating to the pathogenesis, prophylaxis and therapy of mumps-associated disease processes.

A persistent infection was initiated by inoculation of a dilute mumps virus preparation into BHK-21 cells (BHKpi). The purpose of this study was to understand the persistence by means of the following:

i. Characterization of the BHK-21 cell culture persistently infected with mumps virus.

ii. Characterization of the virus released from persistently infected cultures by comparison of its properties to the virus used to initiate the persistence.

iii. To attempt to understand the virus-cell interaction; i.e., the host-parasite relationship in the persistence.

II. Literature Review

A. Historical Background

> "In Thasus, about the autumnal equinox, and under the Pleiades, the rains were abundant, constant, and soft, with southerly winds; the winter southerly, the northerly winds faint, droughts; on the whole, the winter having the character of

> spring. The spring was southerly, cool, rains small in quantity. Summer, for the most part, cloudy, no rain, the Etesian winds, rare and small, blew in an irregular manner. The whole constitution of the season being thus inclined to the southerly, and with droughts early in the spring, from the preceding opposite and northerly state, ardent fevers occurred in a few instances, and these very mild, being rarely attended with hemorrhage, and never proving fatal. Swellings appeared about the ears, in many on either side, and in the greatest number on both sides, being unaccompanied by fever so as not to confine the patient to bed; in all cases they disappeared without giving trouble, neither did any of them come to suppuration, as is common in swellings from other causes. They were of a lax, large, diffused character, without inflammation or pain and they went away without any critical sign. They seized children, adults, and mostly those who were engaged in the exercises of the palestra and gymnasium; a few women were also affected. Many had dry coughs without expectoration, and accompanied with hoarseness of voice. In some instances earlier, and in others later, inflammations with pain seized sometimes one of the testicles, and sometimes both; some of these cases were accompanied with fever and some not; the greater part of these were attended with much suffering. In other respects they were free of disease, so as not to require medical assistance."
>
> Description of parotitis by Hippocrates
> Epidemics, Book 1, Year 1
> (translated by Adams 1886)[2]

Mumps has been recognized as a clinical entity since the 5th century B.C. Its most common manifestations were clearly recorded by Hippocrates, who described it as a mild epidemic sickness characterized by nonsuppurative swellings near the ears and occasionally accompanied by painful enlargement of one or both testes. In modern times, Hamilton, 1790 (54), was among the first to stress

orchitis as a frequent manifestation of the infection. He also noted certain patients suffering from parotitis who showed symptoms referable to the central nervous system. However, it was not until the beginning of the 20th century that clinically apparent and inapparent meningoencephalitis was generally recognized as a complication. Thereafter, it also became evident that other organs, e.g. the ovary and the pancreas might be injured by the infectious agent (127). During the first several decades of this century considerable effort was expended in attempts to determine the etiologic agent. Various reports appeared incriminating a variety of bacteria as well as a spirochete, but the etiologic agant was not isolated and identified as a virus until 1934, when Johnson and Goodpasture produced parotitis in monkeys by inoculating bacteria-free infectious material directly into Stensen's duct (29). No major progress was made, however, in characterizing the virus and in understanding the pathogenesis and immunological aspects of the disease, until the virus was propagated in the chick embryo and was found to agglutinate chicken erythrocytes, similar to influenza virus. In 1963 Hilleman successfully attenuated mumps virus by passage in chick embryo tissue culture. Known as the Jeryl Lynn strain, this vaccine was licensed in the U.S. in December 1967 (99).

Interestingly canine mumps has also been described by

several investigators (19,107) in such cases in which (a) submaxillary or parotid enlargement was observed and (b) antibody titers to both mumps viral and soluble antigens were detected. Attention has been drawn to the possibility of a canine reservoir of this paramyxovirus.

B. Mumps Virus

1. Clinical Aspects

After an 18 to 21 day incubation period, the disease is characterized by the appearance of parotitis, sometimes foreshadowed by several signs which, during an epidemic, orient the diagnosis: slight fever, some malaise, discomfort upon chewing, and acute unilateral or bilateral otalgia.

Parotid enlargement, at first unilateral, becomes bilateral in one to five days. The swelling is accompanied by a moderately febrile state.

At times other salivary glands become involved, as revealed by the palpable swelling of the submaxillary and sublingual glands.

The evolution of parotitis is benign; after seven to ten days the swelling begins to subside, and it disappears completely without sequel, toward the fifteenth day.

Mode of Transmission. Mumps is transmitted from one individual to another by saliva containing the virus. Transmission may occur by direct transfer, by

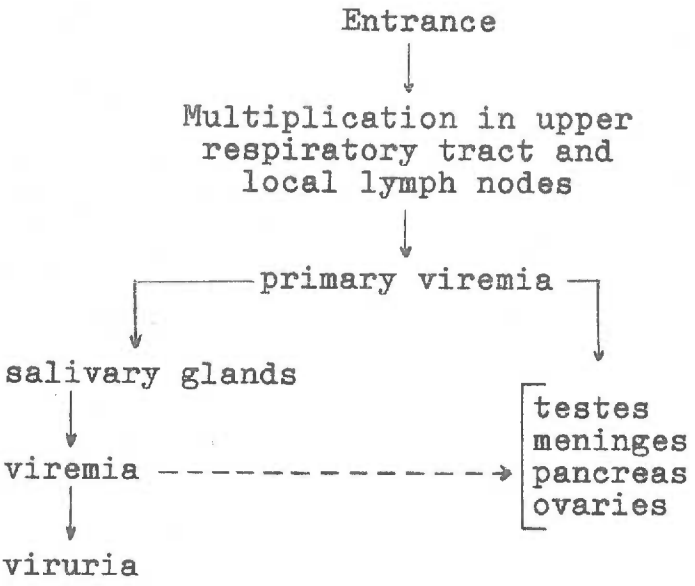
air-suspended droplets, or by fomites recently contaminated with saliva (71).

Pathogenesis. Although the pathogenesis of mumps infection is not yet precisely understood, the evidence available strongly supports the following scheme: Virus first multiplies in an unknown site, presumably the upper respiratory tract; invasion of the blood stream then occurs and infection of the salivary glands and other organs is elicited (see Fig. 1).

Period of Infectivity. The period of communicability, as determined by the isolation of virus from the saliva in naturally occurring mumps as well as in infections experimentally induced, may extend from 6 days before salivary gland involvement until 9 days thereafter (43,52,74). The virus may be present in saliva of subclinical or inapparent infections and cases of orchitis or meningitis in which enlargement of the salivary gland is absent (52,64). Mumps virus is also excreted in the urine for as long as 14 days after onset of illness (120,121).

Mumps virus frequently affects other organs. Its localization can be associated with parotitis or can occur without it. Involvement of the testes announced by a sharp rise in temperature with high fever, is characterized by swelling of the bursula testium. Only the testes increases in volume; the epididymis and the ductus

Figure 1. Schematic representation of the pathogenesis of mumps.



deferens remain normal. Orchitis occurs in about 20% of postpubescent patients.

Central nervous system involvement in its most usual form occurs as "aseptic" meningitis. This does not differ from other virus-induced meningitis. The lymphocyte level of the spinal fluid is often elevated. Serious complications can occur following mumps-induced meningitis. Deafness is fortunately rare but it is one of the most serious complications of mumps.

Numerous studies have attempted to examine the role of mumps virus in encephalitides and even in the persistence of mumps virus in brain tissue (8,49, 59,129). Widespread cytoplasmic inclusions can be observed in neurons, ependyma, and choroid plexus. Optic neuritis has also been described secondary to mumps virus (112). However, studies examining the relationship of mumps virus infections and measured intelligence provided no evidence of intellectual impairment (78).

Involvement of the pancreas may also occur with mumps virus (27,45). It begins suddenly with a rise in temperature, persistent dull epigastric pain, and vomiting. The blood amylase level is above normal, although this increase may occur without clinically evident impairment of the pancreas. In addition, several reports have indicated the possibility of a relationship between primary mumps infection resulting in diabetes mellitus

(28,47,63,106,113). However, to date no concrete etiologic evidence is outstanding.

Mumps virus and its association with arthritis was described initially in 1850 (76). The arthritis may begin 1 day before to approximately 15 days after the onset of parotitis. Mumps-associated arthritis is thought to occur predominantly in postpubertal subjects, and most frequently in the third decade of life. The large joints are most commonly affected, especially the knees, ankles, shoulders, and wrists. The precise etiology of mumps arthritis remains obscure (108).

Other sites of localization of the mumps virus, whether or not associated with parotitis, are much rarer. Thus, involvements of the prostate, epididymis, ovaries, liver, spleen, thyroid, kidneys, heart, and mammary glands have all been described (5,20,44,70,82,109).

It must be stressed here that mumps is such a ubiquitous virus that many subclinical cases with mumps virus are undoubtedly present with no apparent direct consequences; however, as will be discussed below, these exposures may be very important in initiating latent infections which may persist.

Mumps and Pregnancy. A view that mumps in the mother may have effects on the fetus has been entertained for some years with some evidence to support it (10). For example Siegel et al. (100) found a high rate

of fetal deaths and abortions following mumps in the 3rd month of pregnancy, and Johnson et al. (60) have shown that mumps virus can induce hydrocephalus.

Several studies have been made with a hamster-adapted strain of mumps virus (36,66). It was demonstrated that mumps virus could proliferate in the uterus and placenta of the pregnant hamster. In later studies (65,77), intranuclear inclusions as well as specific immunofluorescence pointed to amniotic fluid as the route of transmission of virus to the fetus, with the respiratory tract as the site of entry.

In addition embryonic mumps virus infection in the host may lead to fetal and postnatal growth retardation (110,130). The mechanism of this retardation has not been established.

On the other side of the coin, interestingly, mumps virus has been used in the treatment of certain disease processes, namely human cancer (7). Of 90 patients with terminal cancer of various kinds, treatment was assessed as very good in 37, and good in 42, excluding 11 patients who had been near death. Administration of mumps virus produced few side effects. The initial antineoplastic effect of the mumps virus therapy seemed to occur rapidly, in proportion to swiftness of proliferation of cancer cells.

Prevention and Control. Because subclinical

infections are common, control of infection by isolation is not effective. The disease can be prevented by immunization. Infectious attenuated virus, inoculated subcutaneously, induces an inapparent infection and development of antibody in greater than 95% of antibody-free subjects (children and adults). The attenuated virus is derived by passage of the Jeryl-Lynn strain in chick embryos and then in cultured chick fibroblasts. It has been used since 1968. Infection follows inoculation of the vaccine, but viremia and viruria are not detectable, clinical reactions do not occur, and virus does not spread to exposed contacts. Although the antibody response is not as great as that accompanying natural infections, antibody levels persist for at least 5 years, and the vaccinees are almost uniformly protected upon exposure to mumps infections.

Formalin-inactivated virus also induces antibody production after two subcutaneous injections, and its clinical effectiveness has been demonstrated in control studies. However, antibodies decline 3 to 6 months after immunization and neither the effectiveness nor the persistence of immunity appears to be as satisfactory as that following the live virus vaccine.

The live virus vaccine is of considerable value for susceptible adults in whom the disease is more severe and the complications more frequent.

Passive immunization with gammaglobulin from convalescent serum has been employed to prevent infection after exposure, particularly in men, for whom orchitis is a relatively frequent and severely discomforting complication. Its effectiveness, however, has not been clearly demonstrated.

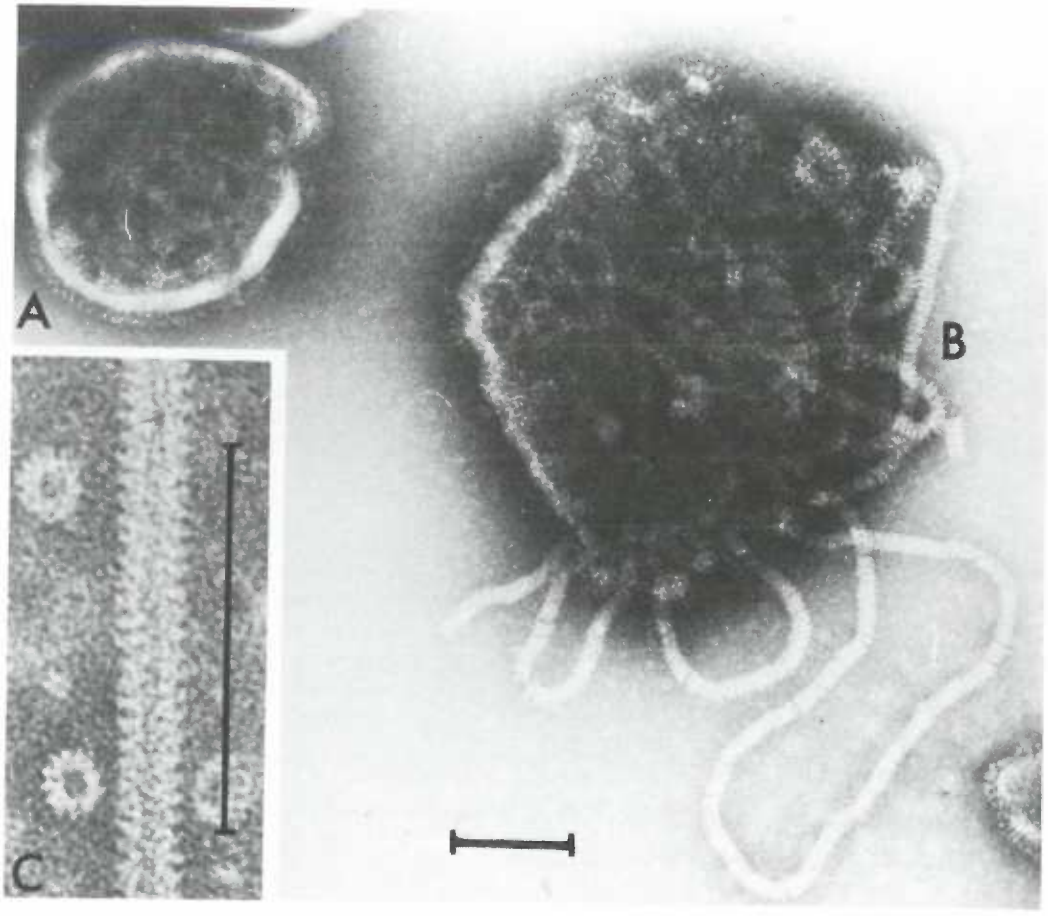
2. Structure and Replication

Mumps virus morphologically resembles other paramyxoviruses (see Fig. 2). It is a pleomorphic virion 100-300 nm in diameter with a helical nucleocapsid 18 nm in diameter (S antigen). The intact virus particle has a lipid envelope about 100 Å thick which is covered with the spiked hemagglutinin (V antigen). The virus is sensitive to ether and chloroform, and infectivity is destroyed at 55C for 20 min. Treatment with formalin destroys infectivity but not the hamagglutinin. The hemagglutinin agglutinates chicken, guinea pig, and human "O" red blood cells at room temperature or 4 C. The hemagglutinin is eluted from red blood cells at 37 C. The single stranded RNA has a molecular weight of approximately 7×10^6 daltons. The nucleocapsid and hemagglutinin are synthesized in the cytoplasm. The site of RNA synthesis is unknown, but the data suggest a cytoplasmic location (26,125).

All strains contain a common complement-fixing viral (V) and soluble (S) antigen. No strain dif-

Figure 2.

Electron micrographs of negatively stained virions of mumps virus (bars=100 nm). (A) Intact virion; peplomers visible at lower edge. (B) Partially disrupted virion, showing nucleocapsid. (C) Enlargement of portion of nucleocapsid, in longitudinal and cross section (Frank J. Fenner and David O. White, Medical Virology)


A
B
C

ferences exist by neutralization with mumps virus antiserum.

RNA and Replication of Mumps and Other Paramyxoviruses. One of the most distinctive fundamental properties of paramyxoviruses, including mumps virus, is possession of the largest genome among viruses which have a single piece of RNA as their genetic material (31). Virion RNA sedimenting at 50S was first found in NDV (3,32,67). Similar RNA has been obtained from SV5 (22), Sendai virus (9), measles virus (98), and mumps virus (34). The 50S RNA molecules isolated from mumps and other paramyxoviruses are thought to represent complete viral genomes, even though they have not been shown to be infectious (67). Lack of infectivity could have a variety of explanations, but the existence of virion transcriptases, and the idea that they are essential in paramyxovirus replication explains the failure of isolated RNA to be infectious.

A peculiar property of the virion 50S RNAs is their ability to self-hybridize. Mumps virus 50S RNA self-hybridized about 20% (34). However, no cross hybridization was obtained when complementary RNA species generated in cells infected with NDV, Sendai, measles, and mumps viruses were annealed with genomic RNA from one of the others (34).

Attachment, Penetration and Uncoating. Viral neuraminidase, an enzyme in search of a function, while considered to play a role in myxovirus release (126)

has also been put forward as an important factor in virus penetration (95). These arguments must be categorized as circumstantial at this point in time.

The idea of "viropexis" or the phagocytic engulfment of virus particles, as a means of virus entry into cells, was first suggested for influenza viruses (35). A number of studies have documented the passage of paramyxovirions into cytoplasmic vacuoles (23,101). This mechanism was questioned early, however, (95,96) and when looked for, fusion of virion envelopes with cell surface membranes has also been observed, leading to the alternative proposal that infection is effected by virus-cell fusion (4, 30, 56).

A number of hypotheses have been advanced about the identity of the "fusion factor" (17,89). The lipid content of virion envelopes and viral hemolytic activity appear to be the parameters most closely related to capacity for cell fusion (51,55). The fusion of virion envelopes with cell membranes appears to require metabolic activity, since artificial membranes, to which enveloped virus attach, have not provided any evidence of fusion (119).

At this time it is not known, on the basis of available evidence whether viropexis, or fusion, or both mediate infection. But as Cohen (21) has pointed out, viropexis does not explain very much, because a virus

particle in a vacuole is still separated by a cell membrane from the cytoplasmic matrix of the cell, and a mechanism must be devised for transport of at least the viral nucleic acid across that membrane. One mechanism for transport out of a vacuole could again be simply fusion, the vacuole membrane behaving like the cell surface membrane. Another possibility, suggested by proponents of viropexis, would involve partial digestion of the virus by lysosomal enzymes (33).

Attempts to trace the fate of virus structural elements in cells by biochemical methods have been hampered by a lack of effective methodology. One major difficulty in this area is to distinguish virions which are nonspecifically degraded by the cell from virions which are engaged in the infectious process. This difficulty may be compounded by the need for high infecting multiplicities to achieve measurable concentrations of isotopically labeled virion components. Virion RNA becomes susceptible to ribonuclease about one hour after infection, but becomes resistant again by about four hours. The first event is attributed to uncoating, and the second to a shift of the uncoated RNA from a single to a double-stranded form. Uncoating in NDV infections (72) was not inhibited by puromycin treatment of the cells, suggesting that new proteins are not necessary for this function. Other observations indicate that nucleocapsids

are only very gradually, and possibly not completely, divested of their protein in infected cells. In addition paramyxovirion transcriptase appears to function *in vitro* in structurally intact nucleocapsids, so it is conceivable that genome transcription at least, and perhaps also genome replication, occur without the RNA being completely uncoated.

Macromolecular Synthesis. One of the distinctive features of paramyxovirus replication that divorces it from the classical scheme of RNA virus replication is the production of large amounts of single-stranded RNA molecules smaller than viral genomes and complementary in base sequences to viral genomes. It is thought that these RNA copies, smaller than genome size serve as mRNA which sediment at approximately 18S (34). Bratt *et al.* (11) found that poly A sequences are added to the RNA transcripts during virion mediated RNA synthesis *in vitro*. Messenger RNA's usually have poly A sequences at their 3' termini and paramyxovirus messenger RNA's conform to this rule.

The 50S RNA in cells appears to be a mixture of genomic RNA and complementary RNA molecules of genome length. The remaining virus-specific single-stranded RNA's appear to be predominantly complementary to viral genomes and they sediment slower than genomes (34). The most abundant of the complementary RNA's is that sedimenting at

about 18S. 22S and 35S species, however, have also been seen (12).

The two polymerase functions, production of 18S RNA (transcription) and 50S RNA (replication) are dissociable by certain manipulations. Mixed infection, for example, with incomplete virus particles seems to inhibit replication more than transcription. A similar result is obtained when protein synthesis is interrupted, as with cycloheximide treatment. Therefore, the presence of at least two enzymes is hypothesized: a polymerase function for 18S production and replicase function for 50S production.

RNA Synthesis Localization. The current status of the site of RNA synthesis is that transcription and replication occur in the cytoplasm and not in the nucleus. The ability of paramyxoviruses to replicate in cells treated with actinomycin D or inhibitors of DNA synthesis rules out a need for continuing nuclear genetic information processing during infection. On the other hand, a small amount of labeled virus-specific RNA is usually found associated with nuclei (Portner & Kingsbury, unpublished data). In addition there is evidence which suggests that a portion of infecting viral genomes rapidly enters the nucleus (16) and there is a period of nucleolar viral RNA synthesis early in infection (15). A nuclear association early in infection has also been demonstrated for NDV by immunofluorescence (62). The

importance a nuclear site might have would be to indicate a requirement for something the cell nucleus could provide, perhaps an enzyme like the cellular transcriptase.

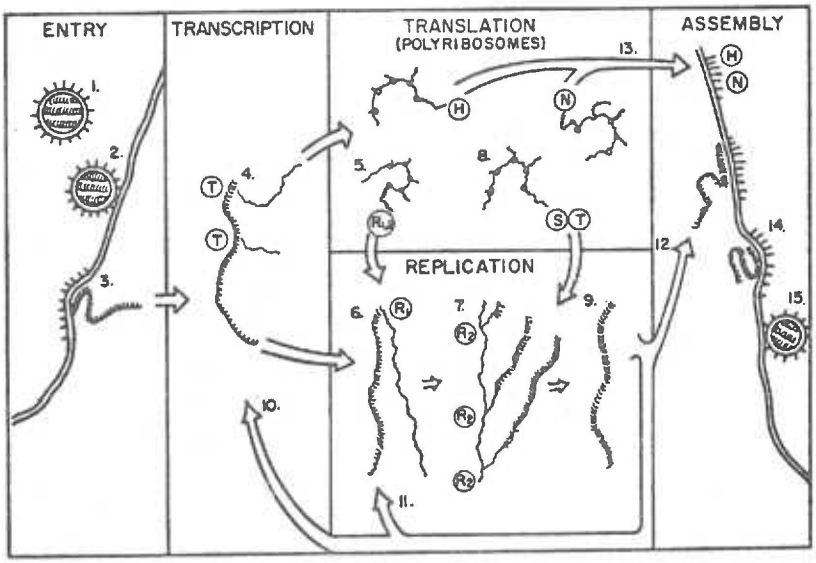
Virus Assembly and Release. Mumps virus and other paramyxovirus nucleocapsids presumably self-assemble like their structural analogs, the helically symmetrical plant viruses (18). The virion envelope proteins, hemagglutinin, neuraminidase and perhaps the 40,000 molecular weight protein, combine with cell membrane lipids (though not necessarily at the cell surface), and modified patches of cell surface membrane containing virion proteins appear. Nucleocapsids align in parallel arrays beneath these modified membrane sites but not elsewhere, suggesting that it is the nucleocapsids which recognize the modified membrane and not vice versa. The membrane then protrudes locally with the nucleocapsids following; there is a constriction at the rear which is pinched off and a new virion is produced.

It must be emphasized that not all of the virions so produced are infectious. There are indications that mumps virus (and other paramyxoviruses) elaborate defective virus particles (34).

Recently Kingsbury (68) has proposed a hypothetical scheme for mumps virus and paramyxovirus replication (see Fig. 3). Although the proposed steps are speculative it encompasses the most important facets of

Figure 3.

A Hypothetical Scheme of Paramyxovirus Replication



paramyxovirus replication and will be discussed here in brief.

A virus particle (1) diffuses into contact with the cell surface membrane, where it attaches via its hemagglutinin (2). The virion envelope fuses with the cell membrane (3), the viral nucleocapsid enters the cytoplasm, and transcription of the genomic RNA begins in the nucleocapsid (4), mediated by the virion transcriptase (T). The RNA template and nascent products form a transcriptive intermediate. The complementary RNA transcripts, smaller than viral genomes, are translated on polyribosomes to give virus-specific replicase(s) (R), hemagglutinin (H), neuraminidase (N), nucleocapsid structure unit (S) and transcriptase (T). Not shown is the protein thought to be involved in virion envelope structure. Also not shown is the possibility that translation might begin before transcription is completed, forming a complex of transcriptive intermediate (in nucleocapsid) with ribosomes. One function of the virion replicase (R) is to make a genome sized complementary strand (6), which, in turn, serves as template for genome replication (7) in a replicative intermediate, via another replicase function (R_2). Nucleocapsid structure units (S) and transcriptase (T) associate with nascent progeny genomes (8). Completed viral nucleocapsids (9) have three possible fates. Some may become involved in transcription (10) which may be indistinguishable from that which occurs

immediately after infection (4). Others may take part in replication (11). The remainder (12) migrate to the cell surface membrane, modified by the incorporation (13) of virion surface proteins (H) and (N). Budding of a new virion commences (14) and a new virion is produced (15).

Polypeptides of Mumps Virus. The polypeptide composition of mumps virus is similar to that observed with other paramyxoviruses as analyzed by polyacrylamide gel electrophoresis (58). There are five to six polypeptides. A large glycoprotein of molecular weight 65,000 to 74,000, a nucleocapsid protein of molecular weight 55,000 to 60,000, a second glycoprotein of molecular weight 52,000,to 56,000, and a low molecular weight polypeptide of molecular weight 38,000 to 40,000. There are also two minor polypeptides present. The heavier glycoprotein has always been the one associated with the hemagglutination and neuraminidase activity. The smallest polypeptide is believed to be a structural protein in the membrane envelope of the virion, because it is a major viral polypeptide and because it can be removed from the virus particle by digestion with detergent and high salt. The function of the smaller glycoprotein and the minor viral polypeptides is as yet unknown (Table 1).

The association of the neuraminidase and hemagglutinating activity with the same polypeptide in mumps virus and other paramyxoviruses is in contrast to

the results with influenza virus, in which these two biological activities were found to be associated with separate polypeptides. The biological function of the smaller paramyxovirus glycoprotein, VP-3, has not been fully elucidated, although it has been suggested that this polypeptide contains the hemolytic and cell fusion properties of the virus.

3. Immune Response to Mumps Virus

Several studies have examined the effect of mumps virus on the immune response (40,42). Mumps infection in the guinea pig model has been shown to depress the expression of pre-existing delayed hypersensitivity (46). This did not occur if the virus was inactivated before administration. In a different study children receiving measles-mumps-rubella vaccine developed an impaired <u>in vitro</u> lymphocyte response to stimulation with antigen (candida) but not with mitogen (phytohemagglutinin and pokeweed mitogen) (83). The impaired response to antigen lasted from one to five weeks after vaccination. There was no alteration in the number of either total or thymus-derived lymphocytes in the peripheral blood after vaccination. This suggests that viral vaccination causes a depression of lymphocyte function rather than a depletion of functional lymphocytes.

C. Persistent Viral Infections and ts Mutants

Paramyxoviruses readily establish persistent

Table 1. Mumps Virus Polypeptides.

Viral polypeptide	Mol. Wt. (d)	Composition	Function
VP-1	66,000	Glycoprotein	Hemag, Neur
VP-2	58,000	Protein	Nucleocapsid protein
VP-3	56,000	Glycoprotein	Unknown, possibly hemolytic & cell fusion
VP-4	48,000	Protein	Unknown
VP-5	44,000	Protein	Unknown
VP-6	40,000	Protein	Structural protein in envelope

infections *in vitro* (50, 87, 97, 116, 124). The significance of the study of *in vitro* persistent infections as model systems for *in vivo* persistent infections and disease processes has been recognized for several years (1,53,61).

Walker, in a review of the viral carrier state in animal cell cultures, has analyzed a number of persistent viral infections by asking a series of very pertinent questions (123). The questions which Walker attempts to answer, to categorize *in vitro* persistent infections, are:

1. Must antibody or other anti-viral factors be supplied in the culture medium to maintain equilibrium in the carrier culture?
2. Can the cultures be freed of virus (cured) by serial cultivation in a medium containing anti-viral antibody, or can virus-free clones readily be obtained by cloning under antibody?
3. Is the culture resistant to superinfection by the carried virus, and is it resistant to challenge by other viruses?
4. What fraction of the cell population is infected under the conditions in which the carrier state is most stable?
5. Do infected cells divide and grow into infected clones?

Using these criteria to differentiate various

types of persistent infections Walker classified a number of *in vitro* persistent infections into four categories. The first category consists of those infections which are comprised of genetically resistant cells (see Table 2). This type of culture is one in which most cells are genetically quite resistant to the carried virus and the infection is perpetuated in a minority of cells in the population. Examples of infections of this kind are those of Coxsackie A9 virus in HeLa cells and poliovirus in HeLa cells.

The second type includes cultures in which the cells are genetically susceptible to the carried virus, but the transfer of virus from cell to cell is limited by antiviral factors in the medium. The characteristics of this group are listed in Table 3. The best examples of this category of infection are found in the cultures of herpes simplex virus in various cell lines.

The third type of infection is that in which the cells are genetically susceptible, but most cells are uninfected and are made resistant to infection by interfering factors produced within the culture. The features of this category are shown in Table 4. Examples of this kind are Newcastle disease virus in L cells and Western equine encephalitis virus in L cells.

The fourth category of infection includes a group of cultures quite different from the other three.

The characteristics are listed in Table 5. Quite a number of viruses, particularly ones of the paramyxovirus group, initiate infections of this type. They have been shown to establish in cell cultures a cell-virus relationship in which there is a cytoplasmic infection of all or a very large fraction of the cells without evident cytopathic effect and in which transmission of the infection is from cell to daughter cell through mitosis. Some of the infections of this type are: measles virus in HeLa cells, HA2 virus in HeLa cells, HA1 virus in human conjunctiva cells, and SV5 virus in primary monkey kidney cells.

Although temperature-sensitive (ts) mutants of mumps virus have not been previously described, temperature-sensitive mutants of several other viruses have been previously reported. Chemically induced ts mutants have been reported in a number of virus classes. For example, ts herpesvirus (134), reovirus (38), adenovirus (128), polio virus (24,25), respiratory syncytial virus (41), influenza virus (75, 79, 104), Semliki Forest virus (114), Sindbis virus (13,14), and vesicular stomatitis virus (VSV; 94), have been used to study viral biochemistry and genetics. Spontaneous ts mutants also occur at a low frequency in many, if not all, virus populations. Spontaneous ts mutants have been observed with reovirus (38), influenza virus (104), VSV (39), and New-

Table 2

Characteristics of Persistent Infections in Cultures of Genetically Resistant Cells

1. Only a small fraction of the cell population is infected. The virus cycle is a standard one in infected cells and the cells are destroyed. Infected cells do not divide or grow into infected clones.

2. Most cells are uninfected and are protected because they are genetically relatively resistant. The culture is relatively resistant to superinfection with homologous virus and the resistance is retained in cured cultures and in uninfected clones.

3. The culture can be cured by antiviral serum in the medium. Clones of uninfected cells are easily obtained by cloning cells in an antibody containing medium.

4. Antibody or other antiviral factors need not be supplied in the medium. Interfering factors are not essential to cultural stability.

Table 3

Characteristics of Persistent Infections in Cultures of Genetically Susceptible Cells Protected by Antiviral Factors in the Medium

1. Only a small fraction of the cell population is infected. The virus cycle is a standard one in infected cells and the cells are destroyed. Infected cells do not divide or grow into clones.

2. Most cells are uninfected and are protected by antiviral factors in the medium. If antiviral factors are removed from the medium, the culture is not resistant to superinfection by the carried virus, and it is not resistant to unrelated viruses. Clones of uninfected cells are easily obtained by cloning cells in an antibody-containing medium.

3. Antibody or other antiviral factors must be supplied in the culture medium. Interfering factors are not essential to cultural stability.

Table 4

Characteristics of Persistent Infections in Cultures of Genetically Susceptible Cells Protected by Interference and Interferon

1. Only a small fraction of the cell population is infected. The virus cycle in infected cells tends to be an abortive one that yields incomplete virus and interferon in addition to some infectious virus. Infected cells do not divide or grow into clones.

2. Most cells are uninfected and are protected by a resistance induced by incomplete virus or interferon. The culture shows increased resistance to superinfection by the carried virus and to infection by other viruses. The increased resistance is lost after the culture is cured and is not found in uninfected clones.

3. The culture can be cured by use of antiviral serum in the medium. Clones of uninfected cells are obtained by cloning in an antibody-containing medium.

4. Antibody or other antiviral factors need not be supplied in the medium.

Table 5

Characteristics of Persistent, Noncytocidal Infections Transmitted Through Cell Division

1. All, or a large fraction, of the cells are infected.
2. Infected cells divide and grow into infected clones. Most clones of cells obtained from the culture are infected.
3. The culture is resistant to superinfection by the infecting virus but is not resistant to unrelated viruses.
4. Antibody or other antiviral factors need not be supplied in the culture medium.
5. The culture is not cured by addition of antiserum to the medium.

castle disease virus (NDV; 92).

Interest in ts mutants has to a large degree been limited to the potential of some of the viruses to function as live virus vaccines. Attempts to use ts mutants of influenza virus (79,84,85), respiratory syncytial virus (41), and herpesvirus type 2 (134) for vaccination of human volunteers have met with limited success. However, there is increasing evidence that naturally selected ts mutants may be involved in disease states in vivo.

Persistent viral infections have been established using cells originating from many different tissues and species, and with viruses from almost every taxonomic grouping (1,123). Studies of the viruses released from persistent infections demonstrated that they often differed from the initiating virus in biological properties, such as virulence, plaque size, antigenicity, heat lability, and timing of the replicative cycle. Mumps virus released from carrier cultures of human conjunctival cells (124) or from BHK-21 cells was less virulent than wild-type mumps virus for several types of cells. Studies regarding the nature of persistent measles virus infections in cell culture have also shown that such infections result in the selection of viral mutants. Measles virus from cultures of diploid human embryonic lung (LU 106) cells banded differently from wild-type virus in cesium chloride gradients (87). HeLa cells persistently infected

with measles virus produced virus with a slower growth rate and lowered virulence as compared to wild-type virus (6, 80, 97); in addition, virus isolated from several HeLa cell carrier cultures produced smaller plaques on susceptible Vero cells than wild-type virus (6, 81).

Youngner and colleagues have intensively studied a line of mouse L cells (L_{NDV}) persistently infected with NDV, a paramyxovirus (90-92, 117, 118). Virus isolated from L_{NDV} cultures (NDVpi) produced smaller plaques on primary chick embryo (CE) cells and was also less virulent *in vivo* for mice and embryonated eggs than the wild-type virus, NDVo. NDVpi also differed from the wild-type virus in a number of other biological properties, including thermal stability of both hemagglutinin and neuraminidase, and in growth pattern in L cells. Youngner *et al.* also showed that NDVpi clones were naturally selected ts mutants which had evolved during the establishment and maintenance of the persistent infection (90). NDVpi did not replicate in primary CE cells or in bovine (MDBK) or canine (MDCK) kidney cell lines at 42-43 C, while NDVo produced near normal yields at that temperature. Biochemical characterization of the NDVpi mutants demonstrated that all NDVpi clones isolated did not synthesize virus-specific RNA (RNA-) at the nonpermissive temperature, and appeared to have defects associated with RNA polymerase activity (91). Further work in Youngner's lab-

oratory showed that the plaque-type marker and the ts marker were independent mutations, and that ts revertant viruses which had lost the ts marker but which retained the small plaque phenotype could be isolated from plaques produced at the nonpermissive temperature (91).

In addition to Youngner's work, an increasing number of reports have indicated that virus recovered from many different types of persistent infections has an impaired ability to replicate at elevated temperatures. Simizu and Takayama have studied a plaque-type mutant from a line of Fructo mouse sarcoma cells persistently infected with Western equine encephalitis (WEE) virus (102). This virus was attenuated in virulence for mice and was also ts at 42 C (103). In contrast to the results with NDVpi, temperature shift experiments suggested that the ts WEE virus mutants might be defective in a late stage of virus maturation at the non-permissive temperature (103).

Other reports have also associated ts virus mutants with establishment of persistent infections. In BHK-21 cell cultures persistently infected with Sendai virus, viral maturation was normal at 31 C but was ts at 37 C (86). Several reports also link persistent measles virus infections <u>in vitro</u> with ts mutants. Virus production in LU 106 cells persistently infected with measles virus was greatly increased at 33 as compared to 37 C (87). In

hamster embryo fibroblasts persistently infected with measles virus, virus maturation was increased 100-fold by growing the cells at 33 C, rather than 39 C (69). Haspel et al. confirmed that maturation of measles virus in chronically infected hamster embryo fibroblasts was temperature-sensitive (48). Armen et al. (6) also demonstrated that measles virus released from chronically infected HeLa cells was temperature-sensitive.

It appears then that the appearance of temperature-sensitive mutants released from persistent viral infections in vitro is not limited to any particular class of virus or type of cultured cells, and the selection of ts mutants may arise, at least in certain circumstances, because of the presence of spontaneous ts viruses in the wild-type population. For example, Valentine (122) described a commonly occurring spontaneous mutant of bacteriophage Qβ, which caused persistent infection of its bacterial host. Virus isolated from the turbid plaques produced by this mutant was strongly ts at 41 C due to defective RNA polymerase production at the elevated temperature.

The selection of temperature-sensitive mutants also occurs during persistent viral infections in vivo. This is the overriding importance of the study of the role of ts mutants arising from persistent viral infections in vitro.

A virus designated "6-94" was isolated from cell cultures obtained by lysolecithin-induced fusion of CV-1 monkey kidney cells with brain cells from a patient with multiple sclerosis (115). The virus, which was similar biochemically to Sendai virus, produced much higher yields at 33 C than 37 C, while yields of Sendai virus, the putative wild-type virus, were equal at both temperatures (73). Temperature-sensitive mutants of reovirus have also been associated with chronic degenerative brain disease induced in laboratory rats (37). Furthermore, plaque production by strains of foot-and-mouth disease virus isolated from carrier cattle (2-12 months after exposure to the virus) was inhibited at 40-41 C, whereas plaque production by vesicular isolates from the acute infection was not affected by the elevated temperature (111).

The mechanisms involved in persistent viral infections that have been proposed include interferon, antibody, genetically resistant cells, ts mutants, defective-interfering (DT) particles, and DNA intermediates (53,93,105,116,123,131). Clearly, DI particles appear to function in the regulation of many persistent infections (53,57,88). Interferon has been reported to be involved in the maintenance of certain persistent viral infections <u>in vitro</u> (16). DNA intermediates have also been reported in cells chronically infected with viruses (105,131,132, 133). However, the interplay of selective pressures within

the persistent infection leading to the most stable virus-cell interaction is still not clearly understood. Further, the increasing evidence that selection of virus mutants, particularly ts mutants, is involved in the initiation or maintenance of persistent infections is too strong to be considered a random process.

The mechanism of selection of ts virus mutants in persistently infected cells is as yet unknown. The frequency of spontaneous ts mutants in NDV populations appears to be relatively high (91). Selection of these virus clones may be involved in the initial establishment of the persistent infections. A continual rheostatic-type evolution of the virus might then proceed through periods of virus synthesis until a stable carrier culture is reached (93). In addition, other factors such as interferon or defective particles may be involved in regulation of virus synthesis in any particular persistent infection.

D. Persistent Infections with Mumps Virus

The studies of persistent infections in tissue culture with mumps virus have come in large part from the laboratories of the Henle's and from Walker and Hinze (50,124). Table 2 summarizes the properties of mumps virus persistent infections in four different cell lines, including the results of this investigator.

Between 80 to 90% of the cells show evidence of infection by either FA or Had with the conjunctiva ar BHK

persistent infections. However, the BHK system releases only a fraction of the virus released in the conjunctival cell system. It is interesting to note that the doubling time of the BHK persistence was less than that of control cells; this is in contrast to the growth rate of the Walker-Hinze system in which the doubling time was reported to be the same as for the control cells.

Immunofluorescence studies showed a unique feature of the BHK persistence, namely that a clear nuclear involvement was observed. It is maximally seen shortly after subculturing and gradually fades as fluorescence is seen developing in the cytoplasm.

Temperature-sensitive mutants of mumps virus were observed being released from the BHK persistence. True temperature-sensitivity experiments were not conducted in the previously reported infections; however, in the conjunctiva persistence shift-up experiments indicated that ts mutants may have been present since this inhibited virus multiplication.

Table 6. Properties of mumps virus persistent infections.

	INVESTIGATORS			
	Henle et al.		Walker & Hinze	Truant & Hallum
Cell system	MCN (human bone marrow)	Lung-To (human embryonic lung)	Human conjunctiva cells	BHK-21
Cell passage # reported	12	12	100	60
% multinucleate cells	ND	ND	ND	40-50%
% infected by HAd or FA	ND	ND	80-95% (FA)	80-90% (HAds or FA)
% infectious centers	ND	ND	ND	5-10%
Released virus	10^2 $TCID_{50}$	$10^{3.2}$ $TCID_{50}$	10^4-10^6 $TCID_{50}$	10^2 FFU/ml (fluorescent focus forming units)
Cell doubling time	ND	ND	Same	BHKpi < BHK
Cell clones	ND	ND	ND	Clones varied from 0-80% showing HAd & FA
Interferon	ND	ND	ND	No detectable IF

(Table 6. cont'd)

	Henle et al.		Walker & Hinze	Truant & Hallum
Cell system	MCN (human bone marrow)	Lung-To (human embryonic lung)	Human conjunctiva cells	BHK-21
Homologous & heterologous interference	Both persistent infections were highly resistant to VSV		Resistant to mumps virus challenge. No resistance to VSV. Low degree of resistance to NDV or Sendai.	Homologous interference. Partial heterologous interference with NDV or VSV.
Temp. sensitivity	ND	ND	ND	MuVpi - ts MuVo - predominantly non ts
Heat sensitivity of virus	ND	ND	ND	MuVpi more heat labile than MuVo.
Nuclear involvement in the infection as shown by FA	Negative	Negative	Negative	Positive; decreases with time in subculture.
Electron microscopy	ND	ND	ND	Cytoplasmic microtubules 180-190Å diam. Intranuclear microtubules, 125Å diam. Corroborated trilaminar membrane structure of wild-type infection(probably assoc. with budding).

(Table 6. cont'd)

	Henle et al.		Walker & Hinze	Truant & Hallum
Cell system	MCN (human bone marrow)	Lung-To (human embryonic lung)	Human conjunctiva cells	BHK-21
UV inactivation profile	ND	ND	ND	Same for MuVo, MuVpi.
Cloning efficiency in soft agar	ND	ND	ND	Control BHK - 2.6% BHKpi - 9.5%
Effect of mumps hyperimmune serum on the persistent infection	Resistance to VSV was greatly reduced with anti-mumps serum treatment. However upon removal of serum, resistance was readily re-established (no cure).		No cure with mumps virus antiserum.	No cure with mumps virus antiserum.
Effect of shift-up and shift-down of cultures	ND	ND	Shift-up - virus multiplication inhibited.	Shift-up - virus multiplication inhibited. Shift-down - no change.
Effect of antibody	ND	ND	Decreased presence of Mu antigens.	Decreased presence of Mu antigens.

ND = not determined.

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Paper 1.

A Persistent Infection of Baby Hamster Kidney-21 Cells with Mumps Virus and the Role of Temperature-Sensitive Variants.

Abstract

A persistent infection of BHK-21 cells with mumps virus (BHKpi) was maintained for over 60 cell passages in the absence of antiserum. Viral persistence was demonstrated in the cultures by hemadsorption, immunofluorescence, multinucleate syncytia, and released mumps virus at the level of 10^2-10^3 fluorescent focus forming units/ml. No detectable levels of interferon were found in cultures persistently infected with mumps virus. Approximately 85-95% of the cells contained viral antigens. Nuclear fluorescence was observed in the persistently infected cells. Mumps virus from persistently infected cultures (MuVpi) was more heat labile than wild-type mumps (MuVo) when subjected to 40 C. BHKpi cells had a more rapid doubling time and a higher cloning efficiency in soft agar in comparison to BHK-21 cells. MuVpi was also found to be temperature-sensitive. The temperature-sensitivity of MuVpi was determined by the efficiency of plating at 33 and 39 C. MuVpi readily established a persistent infection in BHK-21 cells with less cytopathology than MuVo, and released temperature-sensitive virus.

Introduction

Paramyxoviruses readily establish persistent infections *in vitro* (6,16,25,29,35,38,39). The significance of the study of *in vitro* persistent infections as model

systems for *in vivo* persistent infections and disease processes has been recognized for some time (1,17,19). The need for model systems for mumps virus is apparent since the agent has been implicated in clinical disorders such as viral arthritis (32), diabetes (34), primary endocardial fibroelastosis (33) and a number of other organ involvements (11,24,41). In addition, the use of attenuated strains of mumps virus in vaccine preparations raises the possibility that the vaccine virus may initiate a persistent infection in the host.

Persistent infections with mumps virus have been described in L and Lung-to cell cultures (16) and in human conjunctiva cells (38,39). The manner in which these persistent infections are maintained, however, is not clear.

In a recent review, Preble and Youngner (28) have discussed the isolation of temperature-sensitive (ts) mutants from numerous cell cultures and animals persistently infected with paramyxoviruses. Temperature-sensitive mutants of Newcastle disease virus have been shown to be involved in the persistence of L, BHK-21, and MDCK cells (27,42). With the attenuated Schwartz strain of measles virus, Haspel *et al.* (12) demonstrated that the virus released from latently infected hamster embryo fibroblasts was temperature-sensitive. This paper reports on the initiation and characteristics of a persistent infection of BHK-21 cells with mumps virus, and the

possible role of ts variants in establishing and maintaining the infections.

Methods

Cells. BHK-21 cells, clone 13, Vero, BS-C-1, WI-38, and HeLa cells were obtained from the American Type Culture Collection (Rockville, Maryland). RK-13 cells were obtained from E.M. Early (NIH) and human amnion cells were kindly supplied by J.S. Youngner (Univ. of Pittsburgh Sch. or Med.). Cells were grown in Eagle's minimal essential medium (MEM) with Hank's salts, 10% heat-inactivated fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, New York), 0.1% $NaHCO_3$, 100 units/ml penicillin G (potassium) and 100 μg/ml streptomycin sulfate. Cultures were maintained in the above media with 2% FCS. Cultures were incubated at 37 C in a 5% CO_2 atmosphere. Once monolayers were confluent, or after virus infection, the cells were maintained in the above media with 2% FCS. Cell cultures were tested periodically for mycoplasma contamination in complete mycoplasma media as previously described (13,14).

Viruses. The Enders and Jones strains of mumps virus were obtained from the American Type Culture Collection (Rockville, Maryland). A clinical isolate of mumps virus was provided by the Communicable Disease Center (Atlanta, Georgia). Mumps virus stocks were grown by allantoic

inoculation of 7 day old embryonated chicken eggs (Northwest Farms, Portland, Oregon) at 37 C, and harvested four to six days later. Virus stocks were concentrated in an Amicon ultrafiltration unit (Lexington, Mass.) with a XM300 membrane.

Solutions. Calcium and magnesium-free phosphate buffered saline (CMF-PBS) used for cell washings and as diluent for antisera, and calcium and magnesium containing PBS, used as diluent for erythrocytes, were filtered through a 0.22 μ membrane (Millipore Corporation, Bedford, Mass.).

Immunofluorescence. Fluorescent antibody (FA) buffer was obtained from Difco Corporation (Detroit, Michigan). A solution of buffered glycerol which consisted of 9 parts glycerol and 1 part FA buffer (pH 7.4) was used for fluorescent specimen examination.

Guinea pig anti-mumps virus serum (Enders, egg, allantoic) was obtained from Flow Laboratories (Rockville, Maryland) and stored at -20 C. Sheep anti-guinea pig serum conjugated to fluorescein isothiocyanate (FITC) was obtained from Sylvana Corporation (Millburn, N.J.). A 1% solution of sodium azide was prepared and used at a final concentration of 0.1% in antiserum and conjugate.

The specificity of the antiserum and conjugate was determined as follows. Application of antiserum alone or conjugate alone to uninfected as well as mumps-infected cultures resulted in no specific fluorescence. No cross-

reactivity could be detected using FITC-labeled chicken anti-NDV, guinea pig anti-measles virus serum followed by FITC-tagged sheep anti-guinea pig serum, or human anti-measles virus serum followed by FITC-tagged sheep anti-human serum. The activity of the antiserum, specific for mumps virus, could be removed using cultures persistently infected with mumps virus, or purified mumps virus grown in BHK-21 cells, HeLa, or primary chicken embryo fibroblasts (CEF). The mumps-specific activity could not be removed by incubation with uninfected BHK-21, HeLa, or CEF cells. Mumps virus antiserum and sheep anti-guinea pig serum were absorbed with BHK-21, HeLa, and CEF cells prior to use with cell cultures. In addition, uninfected control cells of the same type were used in all staining reactions.

Cytology and histology. Cells grown on coverslips in Leighton tubes or Linbro trays were fixed for 5 min in 95% methyl alcohol and stained with hematoxylin and eosin. The stained cell cultures were observed for morphology, cellular characteristics, and for viral-induced cellular inclusions.

Microscopy. Specimens were observed on a Zeiss GF microscope fitted with a darkfield condenser, type 50 barrier filter, and auxiliary lens type 1. A Reichert illumination unit was used with an Osram HBO 200 W mercury vapor bulb as the light source and a BG-12 excitation filter. A

Zeiss 35 mm camera was used with Kodak Daylight High Speed Ektachrome (ASA 160). Optimal exposure ranged from 60-120 seconds.

Fluorescent focus assay. Fluorescent assay techniques for several viruses have been previously reported (30, 36). In an effort to minimize the time needed to assay mumps virus, indirect immunofluorescence was utilized. Monolayers of HeLa cells were grown to confluency in 10 x 35 mm plastic tissue culture dishes (Falcon Plastics, Oxnard, California). At confluency the medium was aspirated and the cell monolayer was washed with CMF-PBS. Viral dilutions were adsorbed to the HeLa monolayers for 2 hr, rocking at 30 min intervals. An agar overlay was applied which contained 0.25% agar (Bacto-Agar, Difco Laboratories, Detroit, Michigan), 2% heat-inactivated fetal calf serum, 0.1% $NaHCO_3$, and 0.005% diethylaminoethyl dextran in two ml of MEM. After 48 hr at 37 C in a 5% CO_2 atmosphere the agar overlay was gently decanted. The cell monolayer was washed twice and fixed for five minutes with 95% methyl alcohol. Following fixation, guinea pig anti-mumps virus serum (1:10 in CMF-PBS, with 0.1% sodium azide) was applied. A 30 minute incubation at room temperature was followed by three rinses in FA buffer. FITC-conjugated sheep anti-guinea pig serum was then applied (diluted 1:20 in CMF-PBS, with 0.1% sodium azide) for 30 min at room temperature

followed by three rinses in FA buffer. Specimens were then mounted with buffered glycerol, covered with a standard cover glass (22 x 22 mm) and observed. The assay was quantitated using an ocular micrometer.

A linear relationship was demonstrated between the concentration of mumps virus and the number of fluorescent foci observed. The linearity of the dose dependency is illustrated in Fig. 1. A linear regression analysis was carried out on the results of these experiments corroborating the line as drawn.

Figure 2 demonstrates the relative ease with which the focus forming unit is seen under moderate magnification (1120 x). Both infected and uninfected HeLa cells can be observed and fluorescence appeared only in the cytoplasm of the infected cells.

The reproducibility of the assay was confirmed by several independent experiments using different virus samples. Maximum variation from the mean was 4.4%. Samples of the Jones, Enders and clinical mumps virus strains were assayed using the techniques described and fluorescing foci appeared within the same time interval and were easily quantitated.

The fluorescent assay was compared to a conventional mumps virus plaque assay (10) in chick embryo fibroblasts and found to be of equal sensitivity.

Infectious centers. Cell monolayer cultures were rinsed with CMF-PBS, scraped with a rubber policeman, and rinsed in maintenance medium. Cell were either counted and diluted 10-fold to extinction or incubated for 1 hr with mumps virus antiserum, rinsed 3 times and then counted and diluted 10-fold to extinction. Aliquots were then assayed by the fluorescent focus forming assay.

Hemadsorption (Had) and hemagglutination (HA). Hemadsorption and hemagglutination tests were performed by the method of Henle (15) using chicken erythrocytes.

Interferon assay. Supernatant culture fluids from infected monolayers were assayed for interferon in BHK-21 cells by the plaque reduction method as described elsewhere (43).

Thermal inactivation. Virus samples were diluted 1:10 in CMF-PBS and submerged in a water bath at 40 C. Samples were removed at the appropriate times, chilled in an ice bath, and assayed immediately by the focus forming assay in HeLa cells. Control samples were kept at 4 C.

Ultraviolet irradiation. A Sylvania germicidal lamp (G30T8) was used at a distance of 22 cm as previously described (4). Under constant voltage, this provided radiation energy of 4,400 ergs per cm^2 per sec.

Homologous and heterologous viral challenge. Cell monolayers of BHK and BHKpi cells were grown to confluency and the number of cells per dish was determined. The

cultures were innoculated with MuVo, vesicular stomatitis virus (VSV), Indiana strain, or Newcastle disease virus (NDV), Herts strain, at a multiplicity of infection (moi) of 1. NDV and VSV were adsorbed for 1 hr and MuVo for 2 hr at 37 C after which the plates were rinsed with CMF-PBS and incubated in MEM plus 2% FCS. Culture fluids were harvested 8 hr post infection for VSV, 12 hr later for NDV, and 48 hr later for MuVo. NDV and VSV were assayed by plaque formation in chicken embryo monolayer cultures and mumps by the fluorescent focus assay.

Cell growth. The comparative cloning efficiency of BHK and BHKpi cells in agar suspension culture was studied using the method of Macpherson and Montagnier (21). Cell doubling time of BHK and BHKpi cells was determined by plating cells in 10 x 35 mm plastic tissue culture dishes at a density of 2.0×10^4 cells/plate in MEM supplemented with 10% FCS. Duplicate samples were harvested and counted every 24 hr to 120 hr and fresh MEM with 10% FCS was added every 48 hr.

Electron microscopy. Cell monolayer cultures were rinsed twice with CMF-PBS and fixed *in situ* for 1 hr at room temperature with 1.4% glutaraldehyde and 1.5% formaldehyde in CMF-PBS. The cells were removed mechanically from the flask with a rubber policeman and centrifuged at 400 x g for 15 min at 4 C. The pellet was resuspended in CMF-PBS and centrifuged again. The washed cells were

post-fixed in 1% (w/v) osmium tetroxide in CMF-PBS for 1 hr at room temperature. The cells were then washed twice in CMF-PBS before dehydration in a graded series of ethanol (75-95%). Following dehydration, the cells were imbedded in Epon 812 which was allowed to polymerize overnight at 60 C. Sections were cut and examined with a Philips EM-211 electron microscope. (The electron microscope studies were performed in collaboration with R. Brooks of the Dept. of Pathology, UOHSC.)

Results

Establishment of the persistent infection. A persistently infected line of BHK-21 cells (BHKpi) was initiated in the following manner. Seven different cell lines (BHK-21, HeLa, Vero, RK-13, BS-C-1, WI-38 and human amnion cells) were inoculated with a 1:10 dilution of a clinical isolate of mumps virus (moi 0.01). All cultures were incubated at 37 C and within 7 days all of the cell lines showed virus-induced cytopathic effects (CPE). By 30 d post infection, the BHK-21 cells were the only cultures to survive. Some cells remained viable and these slowly increased in number. Multinucleate giant cells were consistently found in the surviving infected cultures, ranging as high as 45-55% of the cells in culture, determined by staing with hematoxylin and eosin. The persistence of mumps virus in the BHK-21 cells (BHKpi)

was shown by hemadsorption of guinea pig or chicken erythrocytes, indirect immunofluorescence, and continual release of mumps virus at the level of 10^2-10^3 FFU/ml. Cultures were passed approximately once a week by treatment with 0.25% trypsin. The trypsin treatment did not affect the degree of hemadsorption, immunofluorescence or the infectivity of released virus from BHKpi cells through the 60th cell passage.

Mumps virus components associated with BHKpi cells. Hemadsorption of BHKpi cells with either guinea pig or chicken erythrocytes demonstrated that as few as 1-5% to as many as 85-95% of the cells carried mumps virus components, depending on cell passage. Hemadsorption could be blocked by pretreatment of the BHKpi cells with mumps antiserum. The percentage of cells showing specific mumps immunofluorescence was consistenly in the range of 85-95%. However, approximately 5-10% of the BHKpi cells formed a fluorescent focus on HeLa cells, indicating that only a small percentage of the cells was actively secreting infectious mumps virus.

In contrast to the exclusive cytoplasmic fluorescence observed in lytic infections of mumps virus (5,40), both cytoplasmic and nuclear immunofluorescence were observed in BHKpi cells. The nuclear fluorescence appeared within one to two days after subculturing and decreased with time in culture, but never disappeared entirely. Cytoplasmic

fluorescence appeared to increase with time in subculture (Figure 3).

By electron microscopy BHKpi cells revealed budding virus particles consistent with mumps virus and the presence of cytoplasmic nucleocapsids which measured approximately 18 nm in diameter. Intranuclear bodies, appearing nucleolar in nature were observed frequently in BHKpi cells and not in control BHK cells. The exact nature of these bodies is being further investigated.

MuVo and MuVpi formed the same size plaque in CEF cells at 33, 37, and 39 C and the two virus populations formed the same approximate size fluorescent focus at either 33, 37, or 39 C. This is similar to chemically induced ts mutants of the Herts strain of NDV which did not have an altered plaque size.

BHKpi growth properties. The comparative growth of BHK and BHKpi cells was studied by planting cells in plastic tissue culture dishes and counting samples at 24 h intervals, replenishing the medium with MEM plus 10% FCS every 48 h. BHKpi cells demonstrated a more rapid doubling time than BHK cells. Maximum differences in growth rate were observed approximately 48 h after subculturing (Table I).

Because of the significant difference in doubling time, the cloning efficiency of BHK and BHKpi cells in soft agar was studied. The difference in cloning ef-

ficiency in soft agar was significantly higher for the persistently infected cells. The BHKpi cells demonstrated a cloning efficiency of 9.5% with a range of 8.9-10.2%. This was compared to a cloning efficiency of 2.6% for the BHK cells with a range of 1.5-3.7%.

In addition, BHKpi appeared to lose much of the original fibroblastic nature of BHK-21 cells. BHKpi cells were observed to be more rounded and overlapping than BHK-21 cells.

Interferon analysis in BHKpi cells. Interferon has been reported to be involved in the maintenance of certain persistent viral infections in vitro (37). No detectable levels of interferon were found when BHKpi cell fluids were assayed by the plaque reduction method on BHK cells suggesting that viral persistence in this system is not maintained by the action of interferon.

Replication of MuVo and MuVpi in BHK cell monolayer culutures. The replication of MuVo and MuVpi was compared in BHK cells. Cultures were infected with virus at an moi of 1. After virus adsorption for 2 h at 37 C, the cells were rinsed twice, maintenance media was added, and virus in the extracellular fluid was harvested at intervals and assayed for released virus. After the cells were rinsed with PBS, cells were freeze-thawed and assayed for cell-associated virus. MuVpi reached a lower maximum titer than MuVo (see Figure 4).

Homologous and heterologous viral challenge. To determine if the persistently infected cultures were resistant to superinfection with homologous or heterologous viruses, MuVo, NDV, or VSV was adsorbed to BHK and BHKpi cells at a moi of 1. The results are given in Table II, and show an inhibition of homologous, and a slight inhibition of heterologous viral challenge.

Thermal and UV inactivation of MuVo and MuVpi. Viruses obtained from certain persistent infections in vitro have been reported to be more heat labile than parental strains (35). MuVo and MuVpi populations were compared for their ability to resist thermal inactivation. The results of a typical experiment, seen in Figure 5, show that MuVpi is more heat labile than MuVo. The difference cannot be attributed to aggregation of virus particles since sonication does not change the shape of the curve. No difference was observed in the rate of inactivation by UV irradiation of MuVo and MuVpi.

Cell cloning. Cell clones were isolated from either agar suspension cultures or from micro-wells and analyzed for the presence of viral components by hemadsorption, immunofluorescence, and released virus. The results are contained in Table III. Clones were obtained which either contained or lacked evidence of mumps virus involvement

as determined by hemadsorption and immunofluorescence. However, no clones were obtained which released infectious mumps virus.

Temperature-sensitivity. MuVpi was determined to be temperature-sensitive. The temperature-sensitivity of MuVpi from BHKpi was determined by the efficiency of plating at 33 and 39 C in HeLa cell monolayers. The efficiency of plating (EOP) was calculated by dividing the titer at 39 C by the titer obtained at 33 C. With MuVo grown in HeLa, BHK-21, CEF or embryonated eggs at 37 C the EOP was approximately 1. MuVpi released from BHKpi cells (cell passage 55) formed very few fluorescent foci at the non-permissive temperature (39 C), while 10^2 FFU/ml were detected at 33 C. When grown in the allantoic chamber of embryonated eggs (37 C), the temperature-sensitivity was maintained (Table IV), suggesting that the phenomenon cannot be attributed to host-controlled variation. If the conversion of one viral characteristic to another occurs in the majority of the viral population after being passed once in a permissive cell type, the variation can be attributed to host-induced modification. This appears not to occur in the case of MuVpi temperature-sensitivity.

Plaque-purified populations of MuVo and MuVpi were analyzed for their EOP. The results (Table V) indicate that most of the MuVpi populations were temperature-sen-

sitive. Interestingly, one of the MuVo plaque-purified populations also demonstrated temperature-sensitivity, suggesting that the persistent infection selects for ts mutants.

BHKpi cells were shifted from 37 C to 33 C. Replicate cell cultures were maintained at 37 C for comparison. The cultures shifted to 33 C were morphologically indistinguishable by light microscopy from cultures incubated at 37 C. Cultures were monitored by hemadsorption, immunofluorescence, and released virus. No significant differences were observed in these parameters with cultures incubated at 33 or 37 C. To determine if there were any differences in viral growth at 33 C as compared to 37 C, plaque-purified populations were amplified in HeLa cells and the released virus assayed at 48 h (37 C). The results (Table VI) demonstrate that there was no significant difference in viral growth at 33 C as compared to 37 C. The temperature-sensitive block appears to be located in the 37 C to 39 C range.

MuVpi and establishment of infection. To determine the effect of MuVpi on establishment of a persistent infection in comparison to MuVo, duplicate samples of both MuVo and MuVpi were used to infect BHK-21 cells at a moi of 0.01. After adsorption for 2 h at 37 C, the cell cultures were fed with maintenance media and incubated at 37 C. At 4 d post-infection the cultures infected with MuVo

had developed approximately 95-100% cytopathology and those infected with MuVpi had developed only 5-10% cytopathology. The progression of the cultures infected with MuVo followed very closely that observed wth the original persistent infection developed some 3 years previously. Some cells remained viable and these slowly increased in number. The progression of the cultures infected with MuVpi was dramatically different, however. Very little cytopathic effect was observed and the culture remained relatively stable. Within approximately 10-14 days post infection all cultures had recovered and were subcultured. This first trypsinization and subculture was designated cell passage 1. The results of hemadsorption, immunofluorescence, and released virus studies are contained in Table VII. The first cell passage demonstrated a very high degree of both hemadsorption and immunofluorescence. The virus released from p1 retained the same EOP as the parent populations. At cell passage 2 the degree of hemadsorption and immunofluorescence observed in cultures infected with MuVo was significantly decreased. Interestingly, the released virus was now ts for all cultures and for subsequent passages.

Discussion

A persistent infection of mumps virus was established in BHK-21 cells. The persistently infected cells demon-

strated that 85-95% carry mumps virus components. No detectable interferon could be found in the culture medium, and both homologous and heterologous interference were demonstrated. BHKpi released 10^2-10^3 infectious mumps virus per ml of culture fluid and approximately 5-10% form infectious centers (see Table VIII). Some of the properties of this persistence are similar to those found in Walker's regulated infections in cell culture (37) since (i) all or a large fraction of the cells are infected when the culture is stable, (ii) the culture is resistant to superinfection by the infectious virus and may show some resistance to related viruses, and (iii) antibody or other antiviral factors need not be supplied in the culture medium in order to maintain an equilibrium. Furthermore, the BHKpi persistence is reminiscent of a mumps persistence in human conjunctiva cells as described by Walker and Hinze, and the L and Lung-to persistent mumps infections of Henle et al. All systems released relatively low levels of infectious mumps virus. The BHKpi cultures produced no detectable levels of interferon. No interferon studies were reported in the previously studied systems of Walker and Hinze or Henle et al. However, both the L and Lung-to cultures were resistant to superinfection by VSV. The conjunctiva-mumps persistence was resistant to superinfection by mumps but not to VSV and was partially resistant to NDV and

Sendai virus. In the BHKpi system, partial inhibition of both VSV and NDV was observed and virtually complete inhibition of MuVo was observed.

Although released interferon was not detected in the culture medium, the resistance of the BHKpi cells to heterologous viral challenge suggests that interferon or an interferon-like mechanism may be functioning. The resistance may have been due to small amounts of interferon, perhaps intracellular, present but not detectable by the assay system. In certain cell cultures a viral resistant state, possibly interferon mediated, can occur before, or in the absence of, detectable amounts of released interferon (2,8).

Several striking differences are apparent in a comparison of the BHKpi persistence to the previously studied systems. In addition to the cytoplasmic fluorescence observed in BHKpi cultures, nuclear fluorescence was also observed. This is in contrast to the exclusive cytoplasmic fluorescence observed in lytic infections with mumps virus (5,40) and the negative nuclear involvement in the cultures of Henle et al. and Walker and Hinze. However, hamster adapted strains of mumps virus have been found to produce intranuclear inclusions in the uterus and placenta of the pregnant hamster (23). The nuclear fluorescence in the BHKpi system appeared within one to two days after subculturing and decreased with

time in culture, but never disappeared entirely. Cytoplasmic fluorescence appeared to increase with time in subculture. The nuclear fluorescence appeared to be nucleolar in nature and suggests the possibility of a nuclear maturation which is associated with the nucleolus during the course of the persistence as suggested by Bukrinskaya (3) for Sendai virus. Nuclear involvement with paramyxoviruses has been previously documented for NDV (18), measles (26), and mumps (23). Furthermore, East and Kingsbury found that actinomycin D severely inhibited mumps virus replication and 50S RNA accumulation (7). It was therefore suggested that continued transcription of the host cell genome is involved in mumps virus genome replication and virion production.

Growth studies, comparing BHK cells to BHKpi cells, showed that the doubling time of the BHKpi was less than that for the uninfected BHK-21 cells. This was in contrast to the growth rate of the cultures in the Walker-Hinze conjunctiva persistence in which the cell doubling times were reported to be the same. Due to the differences in doubling time, the cloning efficiency in soft agar of the persistently infected cells was compared. The cloning efficiency in soft agar was significantly higher for the persistently infected cells. It is unclear at this time why viral infection should increase the growth rate of these cells. Increased cloning efficiency in soft

agar is one of the manifestations of virus transformation as demonstrated by polyoma virus (22) or Rous sarcoma virus (20). This condition is usually regarded as a result of close association of the virus genome with that of the cell. It is of interest that a persistent mumps infection of BHK-21 cells is accompanied by at least one of the parameters characteristic of virus transformation. This is in contrast to a persistent parainfluenza virus infection of BHK-21 cells (9) in which these changes did not occur. Cell clones were obtained which contained varying degrees of mumps virus involvement. However, no clones were obtained which actively secreted infectious mumps virus. That is, all were non-productive. It is not clear why infected clones do not secrete infectious virus. Several possibilities exist. The number of cell clones isolated (18) may not have been sufficient to obtain cell populations which actively secrete infectious virus. This appears not to be the case since 5-10% of the cells form infectious centers. Another possibility is that cells actively secreting infectious mumps virus do not survive the cloning process.

Several mechanisms for establishment and maintenance of persistent viral infections <u>in vitro</u> include interferon, antibody, genetically resistant cells, temperature sensitive or other viral mutants, defective interfering

particles, and DNA intermediates (17,28,31,35,37,44) none of which are mutually exclusive.

In the BHKpi system no interferon could be detected and the persistent infection was established and maintained in the absence of mumps virus antibody. The role of the cell in the virus-cell relationship is very important; however, genetically resistant cells have not been studied in the BHKpi persistence.

By the efficiency of plating at 39 C on HeLa cells, MuVpi was clearly found to be temperature-sensitive. Plaque purified populations of MuVpi (except for MuVpi 205) were also ts. When the incubation temperature of the BHKpi cells was lowered from 37 C to 33 C, no significant differences were found in hemadsorption, immunofluorescence, or released virus. The temperature sensitivity cannot be attributed to heat lability since (1) plaque purified populations of MuVpi can be isolated which do not exhibit ts properties, and (2) no significant difference in EOP can be observed in MuVo populations which are heat labile.

MuVpi readily established a persistent infection in BHK-21 cells with very little cytopathology and also releases virus which is ts. When a persistent infection is initiated with MuVo it appears that a ts variant is selected for early in the process of establishment of the persistent state. It has been shown that subpopulations

of MuVo which are ts are already present and in the process of establishment of a persistent state may be selected for. The mechanism of selection of the mutants by the persistent infection remains unknown. However, emergence of ts variants in persistent infections appears not to be a random process, and these mutants may play a role in establishment and maintenance of the carrier state.

No defective virus particles were observed in the BHKpi persistence. Sucrose gradient centrifugation of ^{3}H-Uridine labeled MuVpi demonstrated a single virus band migrating at a density of 1.19 gm/cc, characteristic of mumps virus. Therefore if defective virus particles are present they are contained in the major virus band. Thus this does not rule out defective virus particles as a possible mechanism of maintenance for this infection. The possible involvement of a DNA intermediate in BHKpi cells is also being investigated. Zhdanov and Parfanovich (46) showed by DNA-RNA hybridization that measles virus DNA transcripts existed in chicken embryo fibroblasts chronically infected with measles virus. Infectious DNA has also been extracted from bovine embryonic kidney cells infected with a ts mutant of respiratory syncytial virus, from Hep-2 cells persistently infected with wild-type respiratory syncytial virus (31), and from Hep-2 cells chronically infected with tick-borne encephalitis virus (45). However, no evidence for DNA intermediates has been found in BHK cells persistently infected with

mumps virus. Transfection studies utilizing DNA isolated from BHKpi cells (at 37 C) did not demonstrate hemadsorption, immunofluorescence or released virus associated with mumps virus (when adsorbed to DEAE-sensitized HeLa cell monolayers). Nevertheless, the presence of defective interfering particles of mumps virus or DNA intermediates of the mumps virus genome and their role in the BHKpi persistence cannot be excluded.

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Table I. Comparative growth rate of BHK-21 and BHKpi cells.

Time (h)	BHK-21	BHKpi
0	2.0×10^4	2.0×10^4
24	3.4×10^4 0.77[a] 31[b]	3.1×10^4 0.63 38
48	1.6×10^5 2.27 11	3.0×10^5 3.27 7
72	2.8×10^5 0.80 30	7.9×10^5 1.40 17
96	8.8×10^5 1.60 15	2.6×10^6 1.70 14
120	2.0×10^6 1.20 20	4.5×10^6 0.80 30

[a] Number of generations for 24 h period.

[b] Generation time (hours).

Table II. Homologous and heterologous challenge of BHKpi with MuVo, NDV and VSV.

Virus	Cell line	Released virus titer	Log_{10} reduction
VSV	BHK	7.7×10^7	
	BHKpi	2.5×10^7	0.48
NDV	BHK	5.4×10^6	
	BHKpi	5.3×10^5	1.01
MuVo	BHK	2.0×10^4	
	BHKpi	5.8×10^1	2.54

Table III. Hemadsorption, immunofluorescence and released virus analysis of BHKpi cell clones isolated from soft agar or micro plates.

Cells	Clone no.	Cell passage tested	% Hemadsorption	% Immunofluorescence	Released virus/ml[c]
BHKpi	Uncloned	52	80-90	80-90	10^2
BHKo	Clones 7-13	2	0	0	0
BHKo	Clones 7-13	5	0	0	0
BHKpi	1[a]	2	1-5	30-40	0
BHKpi	1	5	1-5	85-95	0
BHKpi	2	2	70-80	30-40	0
BHKpi	2	5	1-5	10-20	0
BHKpi	3	2	1-5	70-80	0
BHKpi	3	5	1-5	80-90	0
BHKpi	4	2	1-5	70-80	0
BHKpi	4	5	1-5	10-20	0
BHKpi	5	2	60-70	80-90	0
BHKpi	5	5	ND[d]	ND	0
BHKpi	6	2	0	0	0
BHKpi	6	5	0	0	0
BHKpi	1'[b]	3	0	0	0
BHKpi	1'	5	0	0	0
BHKpi	2'	3	0	0	0
BHKpi	2'	5	0	0	0

(Table III. cont'd)

Cells	Clone no.	Cell passage tested	% Hemadsorption	% Immunofluorescence	Released virus/ml[c]
BHKpi	3'	3	50-60	50-60	0
BHKpi	3'	5	50-60	50-60	0
BHKpi	4'	3	0	0	0
BHKpi	4'	5	0	0	0
BHKpi	5'	3	0	0	0
BHKpi	5'	5	0	0	0
BHKpi	6'	3	0	0	0
BHKpi	6'	5	0	0	0
BHKpi	7'	3	90-100	90-100	0
BHKpi	7'	5	1-10	90-100	0
BHKpi	8'	3	0	0	0
BHKpi	8'	5	0	0	0
BHKpi	9'	3	5-15	5-15	0
BHKpi	9'	5	50-60	50-60	0
BHKpi	12'	3	50-60	50-60	0
BHKpi	12'	5	90-100	90-100	0
BHKpi	13'	3	0	0	0
BHKpi	13'	5	0	0	0
BHKpi	15'	3	70-80	80-90	0
BHKpi	15'	5	90-100	90-100	0

[a] BHKpi clones 1-6 isolated from soft agar.
[b] BHKpi clones 1'-15' isolated from micro plates.
[c] Released virus assayed in HeLa (FFU) and CEF (plaque method).
[d] Not determined.

Table IV. Efficiencies of plating of MuVpi released from BHKpi persistence and MuVpi amplified in embryonated eggs at 37 C.

	Released virus 33 C FFU/ml	Released virus 39 C FFU/ml	Ratio of plating efficiencies 39 C/33 C
MuVpi p55*	1.0×10^{2}	< 10	$< 1 \times 10^{-1}$
MuVpi p1 egg**	2.0×10^{4}	< 10	$< 5 \times 10^{-4}$
MuVpi p2 egg	1.0×10^{5}	6.5×10^{2}	6.5×10^{-3}

*Released virus from BHKpi mumps persistence. Cell passage no. 55

**Mumps virus from first allantoic amplification in embryonated eggs, 37 C.

Table V. Ratios of plating efficiencies (39 C/33 C) of plaque-purified MuVo and MuVpi.

Virus	FFU/ml at 33	FFU/ml at 39	Ratio of plating efficiencies 39 C/33 C
MuVo 101	1.2×10^4	4.7×10^3	0.4
MuVo 102	3.9×10^3	3.8×10^3	1.0
MuVo 104	3.9×10^1	7.8×10^1	2.0
MuVo 105	1.6×10^3	1.2×10^3	0.8
MuVo 107	1.2×10^3	5.8×10^2	0.5
MuVo ts 108	3.9×10^2	<10	$<2.6 \times 10^{-2}$
MuVpi ts 201	7.8×10^2	<10	$<1.3 \times 10^{-2}$
MuVpi ts 202	7.8×10^2	<10	$<1.3 \times 10^{-2}$
MuVpi ts 204	1.6×10^3	<10	$<6.3 \times 10^{-3}$
MuVpi 205	1.9×10^3	1.6×10^3	0.8
MuVpi ts 209	3.9×10^2	<10	$<2.6 \times 10^{-2}$
MuVpi ts 210	1.9×10^3	7.8×10^1	4.1×10^{-2}

Table VI. Temperature-sensitivity of plaque-purified MuVo and MuVpi populations at 33 C and 37 C.

Virus	Temperature (C)	Released virus at 48 h (FFU/ml)
MuVo 8	33	6.8×10^3
MuVo 8	37	7.8×10^3
MuVo 9	33	2.9×10^3
MuVo 9	37	3.9×10^3
MuVo 12	33	2.9×10^3
MuVo 12	37	1.9×10^2
MuVo 13	33	7.6×10^3
MuVo 13	37	7.4×10^3
MuVo 16	33	2.9×10^3
MuVo 16	37	3.9×10^3
MuVpi 52	33	3.9×10^2
MuVpi 52	37	5.8×10^2
MuVpi 54	33	5.8×10^2
MuVpi 54	37	7.8×10^2
MuVpi 55	33	5.8×10^2
MuVpi 55	37	3.9×10^2
MuVpi 56	33	1.9×10^2
MuVpi 56	37	1.9×10^2
MuVpi 58	33	3.9×10^2
MuVpi 58	37	5.8×10^2

Table VII. Hemadsorption, immunofluorescence and efficiencies of plating of BHK cells infected with MuVo or MuVpi.

Virus	% Hemad-sorption	% Immuno-fluorescence	Released Virus 33 C FFU/ml	Released Virus 39 C FFU/ml	Ratio of plating efficiencies 39 C/33 C
Cell passage 1					
$MuVo^{*}_{a}$	100	100	6.8×10^{4}	8.5×10^{4}	1.2
$MuVo_{b}$	100	100	6.8×10^{4}	5.1×10^{4}	0.8
$MuVpi_{c}$	100	100	1.0×10^{3}	<10	$<1.0 \times 10^{-3}$
$MuVpi_{d}$	100	100	7.0×10^{3}	<10	$<1.4 \times 10^{-3}$
Cell passage 2					
$MuVo_{a}$	5	5	6.8×10^{3}	<10	$<1.5 \times 10^{-3}$
$MuVo_{b}$	5	5	3.4×10^{3}	<10	$<2.9 \times 10^{-3}$
$MuVpi_{c}$	95	95	3.0×10^{4}	<10	$<3.3 \times 10^{-4}$
$MuVpi_{d}$	95	95	3.0×10^{4}	<10	$<3.3 \times 10^{-4}$
Cell passage 3					
$MuVo_{a}$	5	5	7.0×10^{4}	<10	$<1.4 \times 10^{-4}$
$MuVo_{b}$	90	5	3.4×10^{4}	<10	$<2.9 \times 10^{-4}$
$MuVpi_{c}$	90	90	6.0×10^{4}	<10	$<1.7 \times 10^{-4}$
$MuVpi_{d}$	90	90	6.8×10^{4}	<10	$<1.5 \times 10^{-4}$

*Two samples each of MuVo and MuVpi were used in this study.

Variation in hemadsorption or immunofluorescence was 5%.

Table VIII. Summary of properties of BHKpi and MuVpi.

Properties of BHKpi	Properties of MuVpi
1. 85-95% carry mumps virus components.	1. More heat-labile at 40 C than MuVo.
2. No detectable interferon produced.	2. Slower replicative cycle than MuVo.
3. Interference with NDV, VSV and MuVo replication.	3. Less virulent than MuVo.
4. Produce 10^2-10^3 FFU/ml.	4. Establishes a persistent infection with less cytopathology than MuVo.
5. Approximately 5-10% cells form infectious centers.	5. Temperature-sensitive.
6. Cytoplasmic and nuclear immunofluorescence.	
7. Doubling time shorter for BHKpi.	
8. Cloning efficiency in soft agar higher for BHKpi.	
9. Uninfected and infected clones isolated.	

Figure 1.

Dose-Response of Fluorescent Focus Forming Assay of Mumps Virus in HeLa Cells

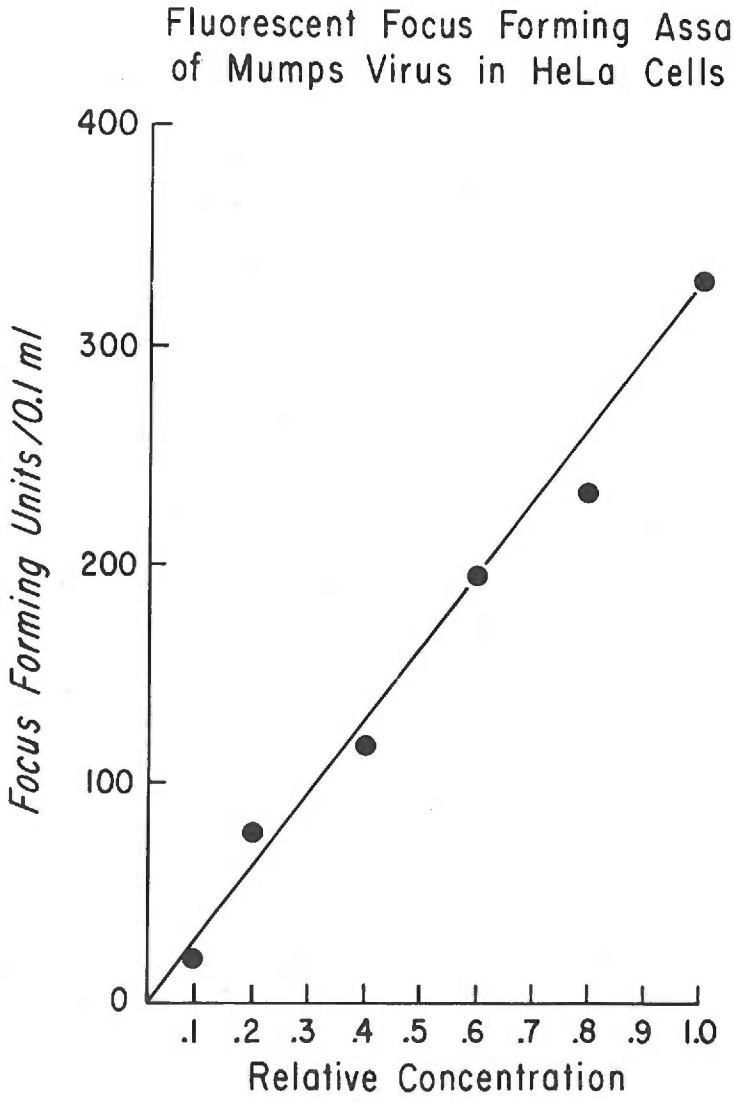

Fluorescent Focus Forming Assay
of Mumps Virus in HeLa Cells
Focus Forming Units/0.1 ml
400
300
200
100
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Relative Concentration

Figure 2.

Mumps Virus Focus Forming Unit (FFU) in HeLa Cell Monolayers. Magnification is 1660X.

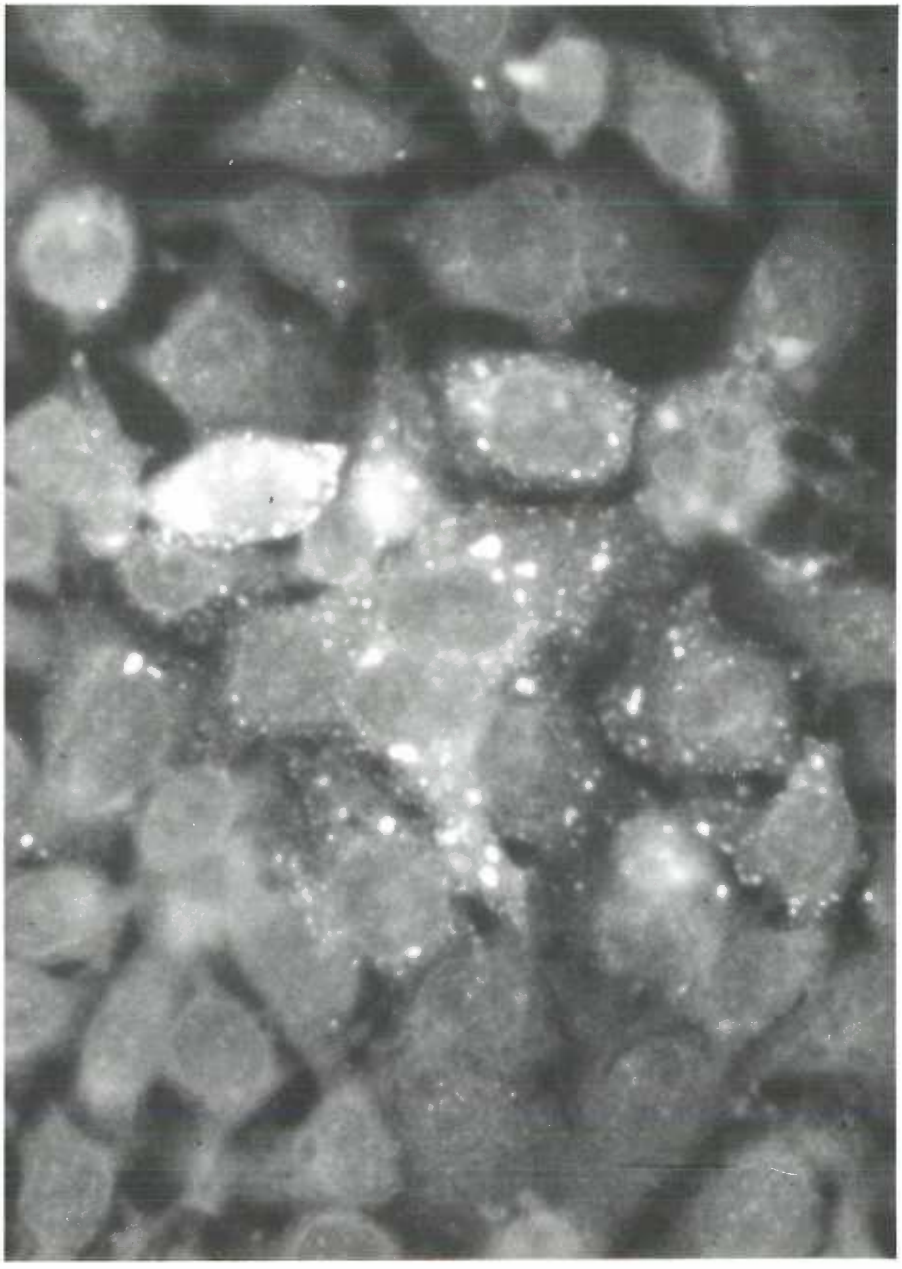
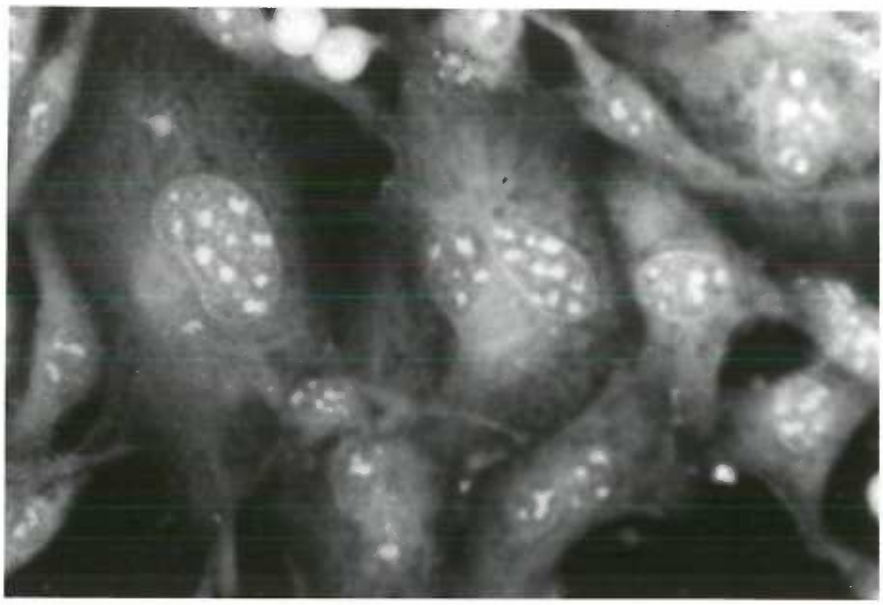
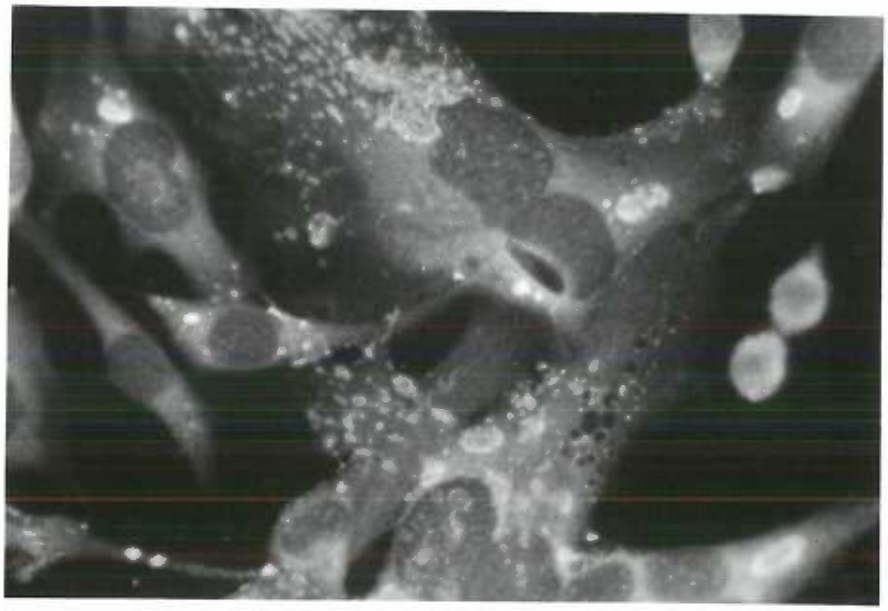


Figure 3.

Mumps Virus Immunofluorescence in BHKpi Cells. (A) Nuclear fluorescence associated with mumps virus components in BHKpi cells (1120X). (B) Cytoplasmic fluorescence associated with mumps virus components in BHKpi cells (1120X).



A



B

Figure 4.

Replication of MuVo and MuVpi in BHK cells. O, MuVo extracellular; △, MuVo cell-associated; ●, MuVpi extracellular; ▲, MuVpi cell-associated.

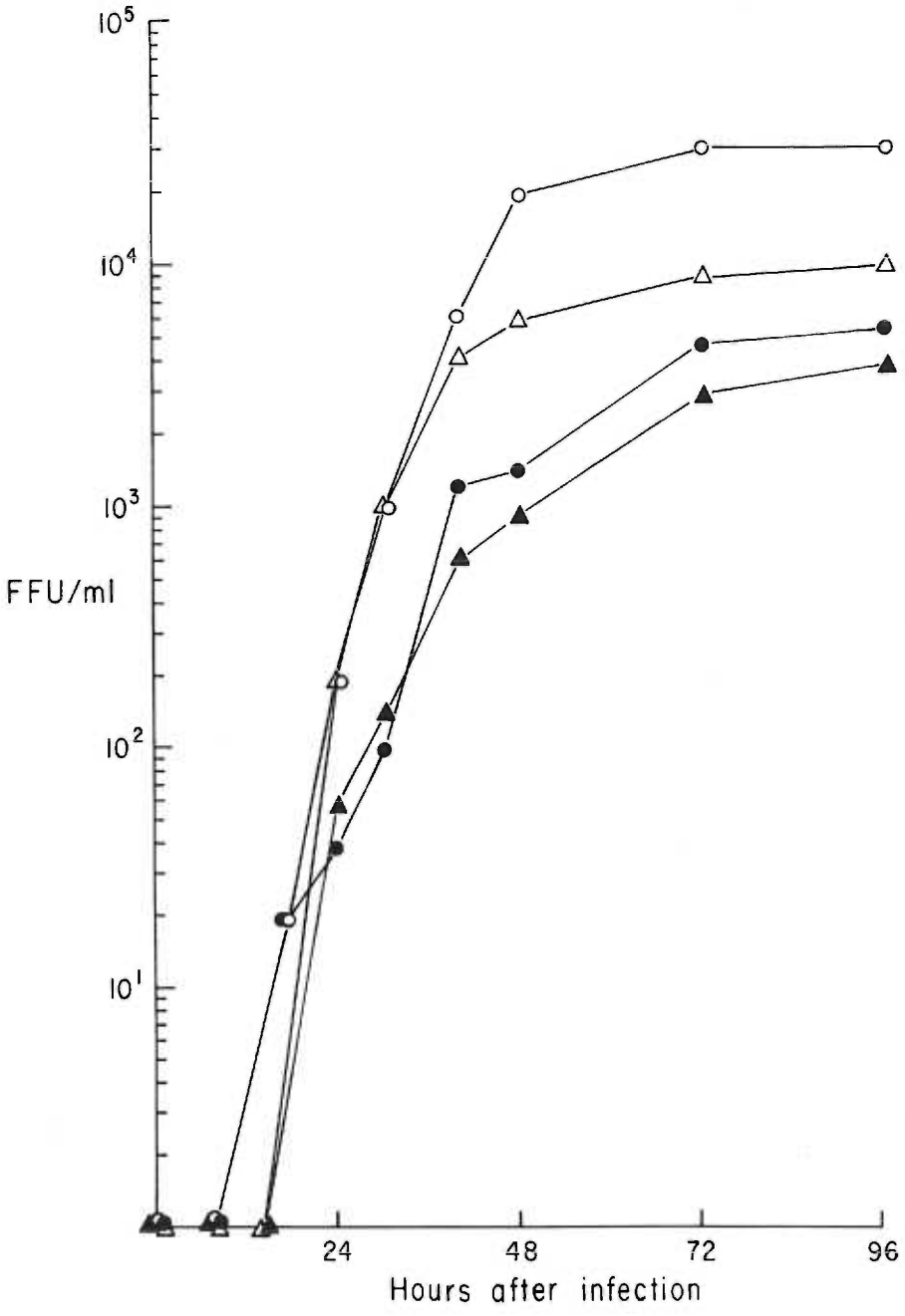
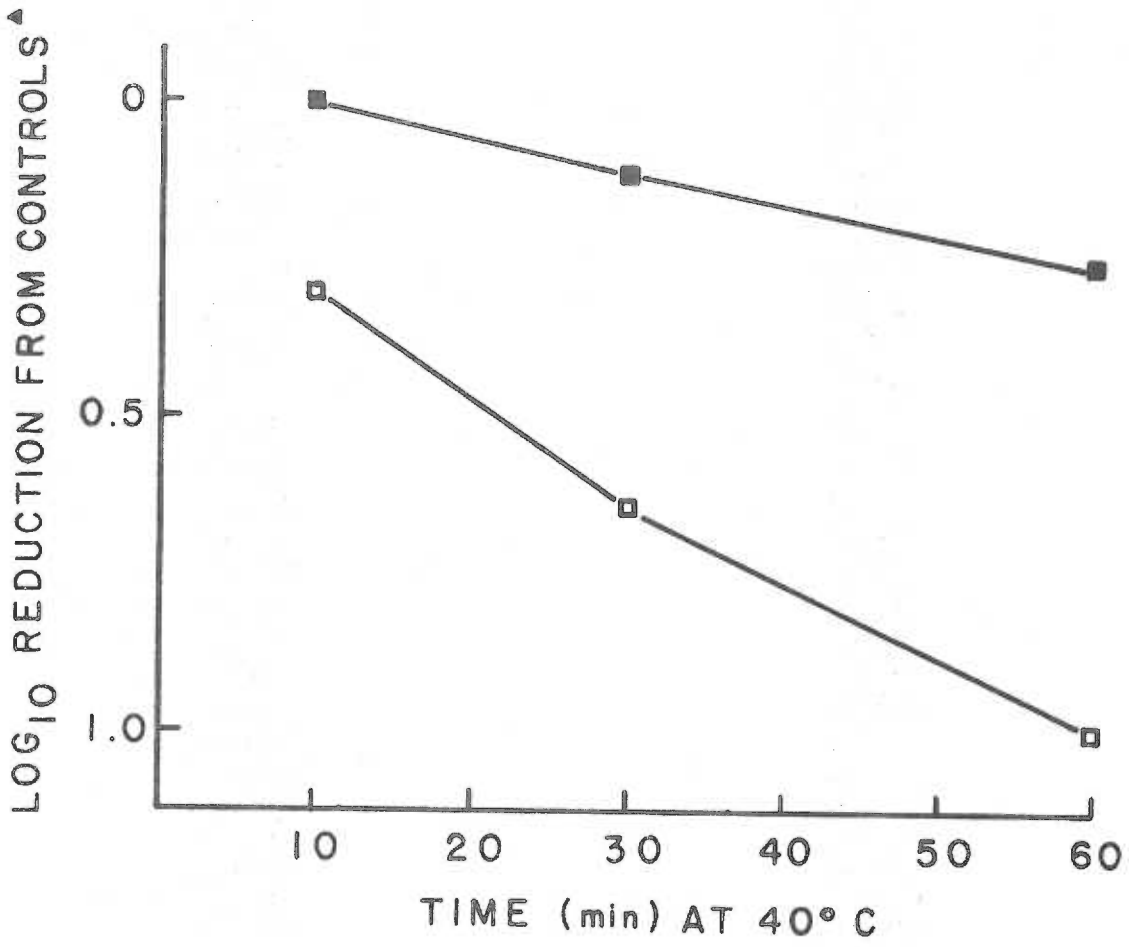

10^5
10^4
10^3
10^2
10^1
FFU/ml
24
48
72
96
Hours after infection

Figure 5.

Thermal Inactivation of MuVo (■) and MuVpi (□) at 40 C.



▲CONTROL SAMPLES WERE KEPT AT 4° C

Paper 2.

Detection of Mumps Virus Antigens in Hddgkin's Disease Tissues

Abstract

Mumps virus (Mu) antigens were demonstrated in biopsied tissues from Hodgkin's disease patients by indirect immunofluorescence. Impression smears from 10 lymph node and 2 spleen specimens revealed viral antigens in the nucleus, cytoplasm or both. Measles virus (M) antigens were detected in six out of seven Hodgkin's disease tissues (lymph nodes) both in the nucleus and cytoplasm. All tissues tested for the presence of Newcastle disease virus (NDV, an avian paramyxovirus) antigens were negative. Control tissues were obtained from patients with non-Hodgkin's lymphomas, breast cancers, adenocarcinomas and a number of other disease processes. In control tissues mumps antigens were detected in seven out of thirty-one specimens and measles antigens in nine out of eighteen tissues.

Introduction

Hodgkin's disease has been considered to be either a neoplasia, an infectious granuloma of unknown etiology, or an immunologic aberration associated with secondary lymphoproliferative response (13). Nonetheless, it is characterized by a progressive and generalized hypertrophy of the lymph nodes which results in tumors which metastasize and is fatal if untreated (21).

In the course of our investigations of persistent

infections by paramyxoviruses *in vitro* (5,7,22,23) we observed in a persistent infection of BHK-21 cells with mumps virus the formation of multinucleate syncytia similar in appearance to Reed-Sternberg cells, which although not pathognomonic, are an essential feature in the diagnosis of Hodgkin's disease (1). This, plus the observation of mumps virus antigens in the nucleus of persistently infected cells *in vitro*, their decreased doubling time and increased cloning efficiency in soft agar over uninfected cells (A.L. Truant and J.V. Hallum, manuscript in preparation), led us to examine biopsy tissues from Hodgkin's disease patients for the possible presence of mumps virus antigens.

Materials and Methods

Fresh impression smears were prepared from either biopsy or autopsy tissues immediately upon dissection. Tissues were tested prior to anatomical pathology examination. The smears were air dried and fixed in 95% methyl alcohol at room temperature for five minutes. They were then reacted with guinea pig anti-mumps virus antiserum (Enders, egg-allantoic, Flow Laboratories) for 30 minutes at room temperature followed by sheep anti-guinea pig antiserum conjugated to fluorescein isothiocyanate (FITC, Sylvana Corporation) for 30 minutes at room temperature. Specimens were observed on a Zeiss

GF microscope fitted with a darkfield condenser, type 50 barrier filter, and auxiliary lens type 1. A Reichert illumination unit was used with a BG-12 excitation filter. Nonspecific fluorescence was minimized by dilution of antisera (mumps virus antiserum 1:10 in phosphate buffered saline (PBS), sheep anti-guinea pig antiserum 1:20 in PBS), counter staining with Evans blue, and absorption of antisera as described below. Reactions were classified as positive when granular fluorescence appeared in the cytoplasm, nucleus, or both.

The specificity of the mumps virus antiserum and sheep anti-guinea pig antiserum conjugated to FITC in the indirect immunofluorescent test was determined as follows. Application of mumps virus antiserum alone or FITC-conjugated antiserum alone to uninfected as well as mumps virus-infected cultures resulted in no fluorescence. No reactivity was detected with mumps virus-infected cultures when incubated with FITC-conjugated chicken anti-NDV, guinea pig anti-measles virus followed by FITC-conjugated sheep anti-guinea pig antiserum, or human anti-measles virus antiserum followed by FITC-conjugated sheep anti-human antiserum. The measles virus antisera used for tissue testing was guinea pig anti-measles virus antiserum followed by sheep anti-guinea pig antiserum conjugated to FITC (as described for mumps virus). Antisera were tested on cultures infected with either wild-type NDV,

M or Mu viruses, and cultures persistently infected with either NDV, M or Mu viruses to serve as positive controls. Absorption of mumps virus antiserum with cultures persistently infected with mumps virus or sucrose gradient-purified mumps virus grown in Baby Hamster Kidney-21 cells (BHK-21), HeLa or primary chick embryo fibroblasts (CEF) removed the anti-mumps virus activity. The mumps virus-specific activity could not be removed by incubation with uninfected BHK-21, HeLa, or CEF cells. Mumps virus antiserum and sheep anti-guinea pig antiserum were absorbed with BHK-21, HeLa, and CEF cells prior to use with tissues.

Results

Mumps virus antigens were detected by indirect immunofluorescence in all fresh Hodgkin's disease lymph node and spleen tissues tested. Table I contains the results of the mumps virus immunofluorescence for all tissues. Clinical diagnoses were corroborated by anatomical pathology examination. In all cases, FITC-conjugated NDV antiserum served as an additional control. Both nuclear and cytoplasmic mumps virus immunofluorescence could be observed in the Hodgkin's disease tissues tested. Cells similar in morphology to Reed-Sternberg cells also expressed mumps virus antigens. Examples of the immunofluorescence are seen in Figures 1-3.

Four non-Hodgkin's disease lymphomas did not demonstrate the presence of mumps virus antigens in biopsied lymph nodes. The integrity of the humoral and cell-mediated immune response in these patients was not known. One out of four tissues derived from patients with breast cancer contained mumps virus antigens; three of these tissues, negative for mumps virus antigens, were breast biopsies, the other tissue was a lymph node. One out of three epidermoid carcinomas and one out of two rhabdosarcomas demonstrated the presence of mumps virus antigens. Lymph nodes from patients with a para-testicular rhabdomyosarcoma, micronodular cirrhosis, dermatopathic lymphadenopathy, malignant teratoma, and a small cell undifferentiated carcinoma also contained mumps virus antigens.

Impression smears prepared from tissues taken from patients with the following conditions were negative for mumps virus antigens: two adenocarcinomas, two melanomas, rheumatoid arthritis, parotid, lung, and kidney cancer, a glioblastoma and an undifferentiated carcinoma. Several tissues taken at autopsy or biopsy from individuals free of any evidence of tumor, were also negative for mumps virus antigens.

A cell line was derived from a trypsinized tissue explant taken from a lymph node of a patient demonstrating mumps virus immunofluorescence (patient #30) and subcultured for nine cell passages. The cell type appeared to

be primarily fibroblastic and through the ninth passage did not reveal the presence of Mu, M or NDV antigens by either hemadsorption or immunofluorescence. Furthermore, no released virus could be detected. Co-cultivation of Hodgkin's disease tissues with Vero cells have failed to induce mumps virus antigens as determined by hemadsorption, indirect immunofluorescence, or released virus.

A measles virus screening of a portion of the tissues revealed that six out of seven Hodgkin's disease tissues demonstrated measles virus-specific immunofluorescence. Six Hodgkin's disease lymph nodes were measles virus-positive and one Hodgkin's disease spleen was measles virus-negative, although all were mumps virus-positive. In some of the Hodgkin"s disease tissues a much stronger measles virus fluorescent reaction was noted in comparison to mumps virus (see Figures 2-3). Similar to the mumps virus immunofluorescence, the measles virus-specific fluorescence could be detected both in the nucleus and cytoplasm. Tissues derived from carcinomas demonstrated the presence or absence of measles virus antigens: one out of four tissues from breast cancers and two out of three tissues from carcinomas contained measles virus antigens. Tissues negative for measles virus antigens included a rhabdosarcoma, non-Hodgkin's disease lymphoma and control tissues. All tissues tested were negative for the presence of NDV antigens by the direct immunofluores-

cent method.

Discussion

The possible infectious nature of Hodgkin's disease has been suggested by epidemiologic evidence. It has been reported that there is a high incidence of clinical onset of Hodgkin's disease in winter (4,25). The finding of seasonal variation in the incidence of the disease is consistent with, but does not prove an infectious agent as the precipitating factor of Hodgkin's disease. Several viral infections produce features of lymph nodes reminiscent of Hodgkin's disease (24). Hence it has been proposed that the Reed-Sternberg cells may originate from virus-induced multinuclear giant cells by malignant transformation (20). A number of different viruses have been reported to be associated with Hodgkin's disease. Schimpff et al. (18) and Gofinnet et al. (8) have reported the increased incidence of Varicella-Zoster infections in Hodgkin's disease patients over other lymphomas. Hehlmann and co-workers reported virus-specific RNA in Hodgkin's disease and other lymphomas (11). In that study sixteen of twenty-four Hodgkin's disease tissues contained RNA which hybridized to the RNA of mouse leukemia virus. C-type RNA viruses have also been detected in tissues derived from Hodgkin's disease patients (12). In addition, elevated antibody levels for Epstein-Barr virus (EBV) have been

reported in patients with Hodgkin's disease as compared to healthy subjects (4,17). However, no EBV DNA could be detected in Hodgkin's disease tissues by DNA-DNA hybridization (16).

Viruses and viral antigens have been found in normal as well as aberrant tissues (2,3,15). Therefore, the mere presence of infectious agents does not suggest an etiology. Infectious agents may be present in diseased tissues due to predisposition by injury or as a secondary involvement due to decreased immunologic competency. Patients with Hodgkin's disease are thought to have impaired cell-mediated immunity (6,9) during the active phases of the disease, which can be restored to normal levels during remission (19). It appears, then that during the depression of delayed-type hypersensitivity in Hodgkin's disease, viral infections may be more easily established than in normal tissues, as seen for other infectious complications of Hodgkin's disease (10). This point may be brought out in the mumps immunofluorescence data presented since, while ten out of ten lymph nodes from Hodgkin's disease patients were positive for mumps antigens, six out of fifteen control lymph nodes were positive; this makes up all but one of the positives in the controls. Perhaps, then, lymph nodes may contain viral antigens and those from Hodgkin's disease patients are more likely to do so.

We have extended these studies into an analysis of the immunologic responsiveness of Hodgkin's and non-Hodgkin's lymphoma patients to mumps virus and other viruses by lymphocyte transformation. Preliminary data suggest that Hodgkin's patients possess a degree of anergy to mumps virus while responding normally to other viral antigens. Non-Hodgkin's lymphoma patients do not appear to demonstrate this restricted anergy. These data will be presented in a future communication.

Whether an infectious agent, possibly a virus, is the etiologic agent of Hodgkin's disease or whether the immunologic impairment permits superinfection with adventitious viruses, or activates a latent viral genome, is unknown.

Table I.

	Patient #	Sex	Age	Disease	Tissue	Immunofluorescence Mumps	Immunofluorescence NDV
Hodgkin's	02	F	23	Hodgkin's	LN*	+	-
	03	F	7	Hodgkin's	S	+	-
	04	unk	unk	Hodgkin's	LN	+	-
	07	M	31	Hodgkin's	LN	+	-
	30	F	20	Hodgkin's	LN	+	-
	31	F	9	Hodgkin's	LN	+	-
	34	M	80	Hodgkin's	LN	+	-
	36	M	16	Hodgkin's	LN	+	-
	39	M	59	Hodgkin's	LN	+	-
	41	F	21	Hodgkin's	LN	+	-
	42	F	14	Hodgkin's	S	+	-
	43	M	unk	Hodgkin's	LN	+	-
Non-Hodgkin's lymphomas	01	unk	unk	Non-HD lymphoma	LN	-	-
	21	F	30	Lymphohistiocytic lymphoma	LN	-	-
	35	unk	unk	Non-HD lymphoma	LN	-	-
	40	unk	unk	Non-HD Lymphoma	LN	-	-
Breast cancer	08	F	62	Breast & bone cancer	LN	+	-
	10	F	51	Breast cancer	B	-	-
	13	F	35	Breast cancer	B	-	-
	15	F	63	Breast cancer	B	-	-
Other	17	M	71	Adenocarcinoma	LN	-	-
	19	F	unk	Adenocarcinoma	B	-	-
	22	unk	unk	Rhabdosarcoma	CC	-	-
	16	M	15	Para-testicular rhabdomyosarcoma	LN	+	-
	37	M	83	Congestive heart failure	LN	-	-
	38	F	unk	Micronodular cirrhosis	LN	+	-

(Table I. cont'd)

	Patient #	Sex	Age	Disease	Tissue	Immunofluorescence Mumps	NDV
Other	33	M	53	Florid reactive hyperplasia	LN	-	-
	32	M	unk	Normal	LN	-	-
	11	unk	unk	Trauma (control)	Syn. cells	-	-
	12	unk	unk	Rheumatoid arthritis	Syn. cells	-	-
	05	M	70	Mycosis fungoides; dermatopathic lymph-adenopathy	LN	+	-
	06	M	21	Malignant teratoma	LN	+	-
	09	M	34	Small cell undiff. carcinoma	LN	+	-
	14	M	65	Undiff. carcinoma	LN	-	-
	20	F	41	Melanoma	Eye	-	-
	24	unk	unk	Melanoma	CC	-	-
	18	M	94	Parotid cancer	Parotid tumor	-	-
	23	unk	unk	Glioblastoma	CC	-	-
	25	unk	unk	Epidermoid carcinoma	CC	+	-
	26	unk	unk	Epidermoid carcinoma	CC	-	-
	27	unk	unk	Epidermoid carcinoma	CC	-	-
	28	unk	unk	Lung cancer	CC	-	-
	29	unk	unk	Kidney cancer	CC	-	-

*LN - lymph node
S - spleen
Syn. cells - synovial

B - breast
CC - cell culture
HD - Hodgkin's disease

Figure 1A.

Impression Smear of Lymph Node from Patient #07 Stained with Mumps Virus Antiserum (1660X).

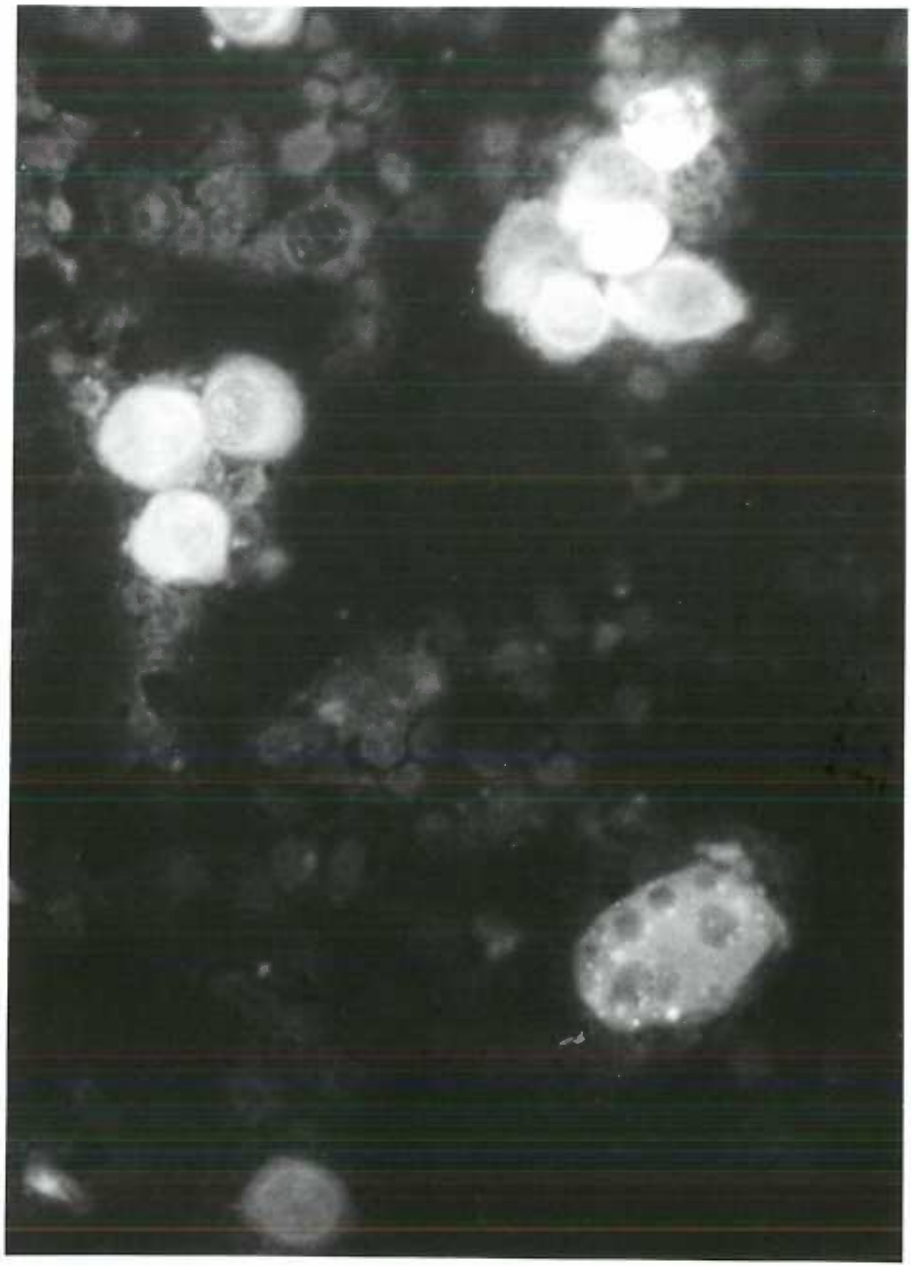


Figure 1B.

Impression Smear of Lymph Node from Patient #07 Stained with Mumps Virus Antiserum (1660X).

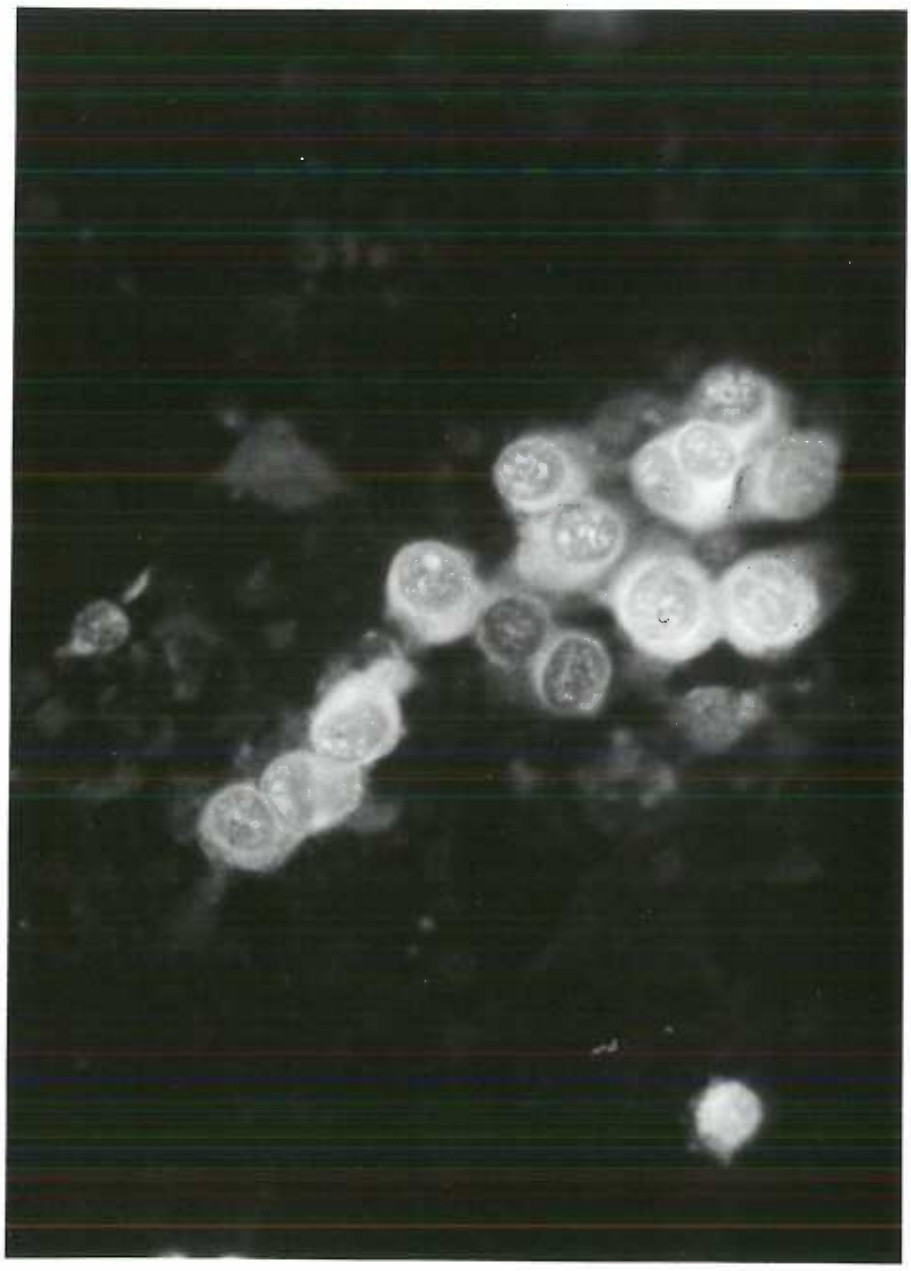


Figure 2.

Impression Smear of Lymph Node from Patient #30 Stained with Mumps Virus Antiserum (1660X).

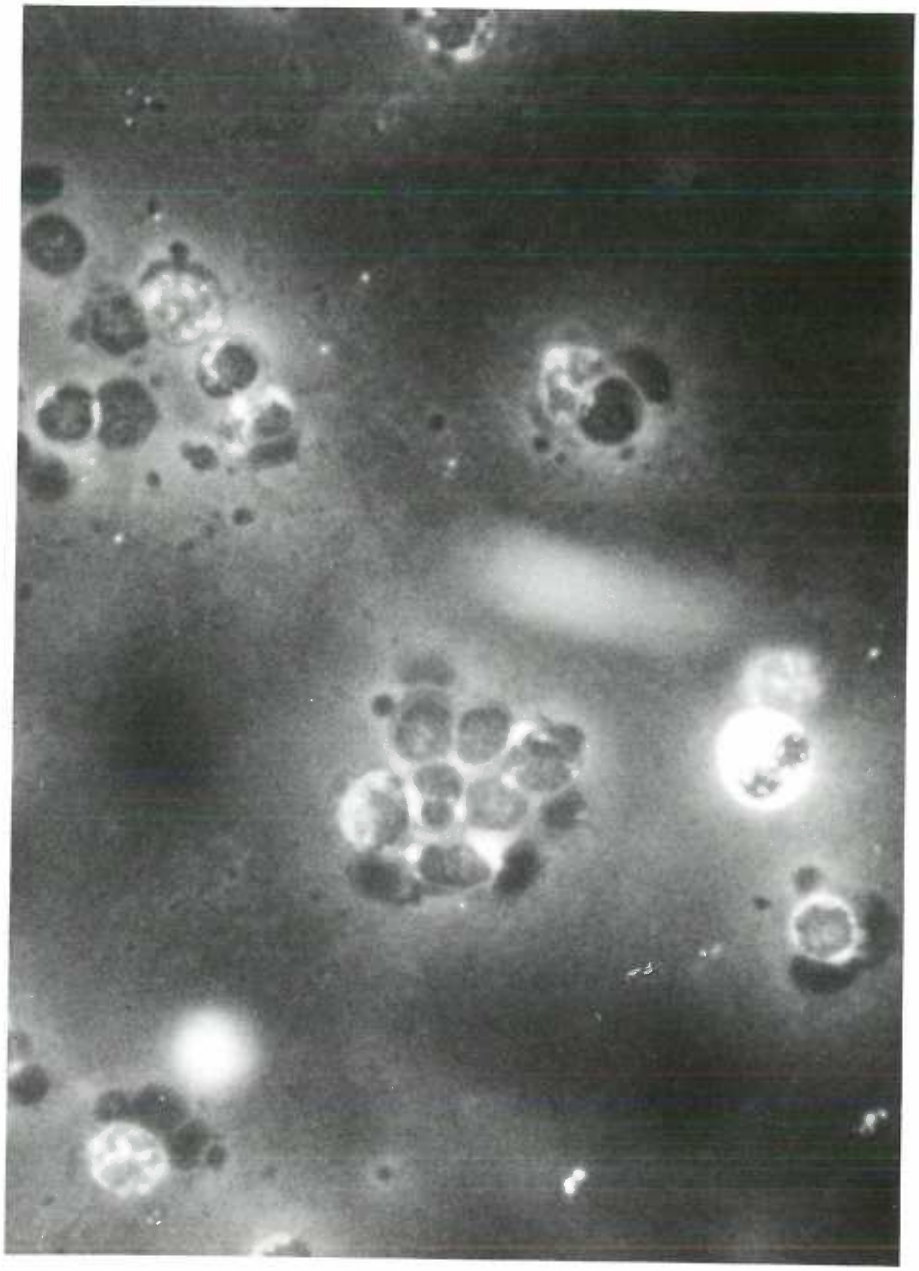
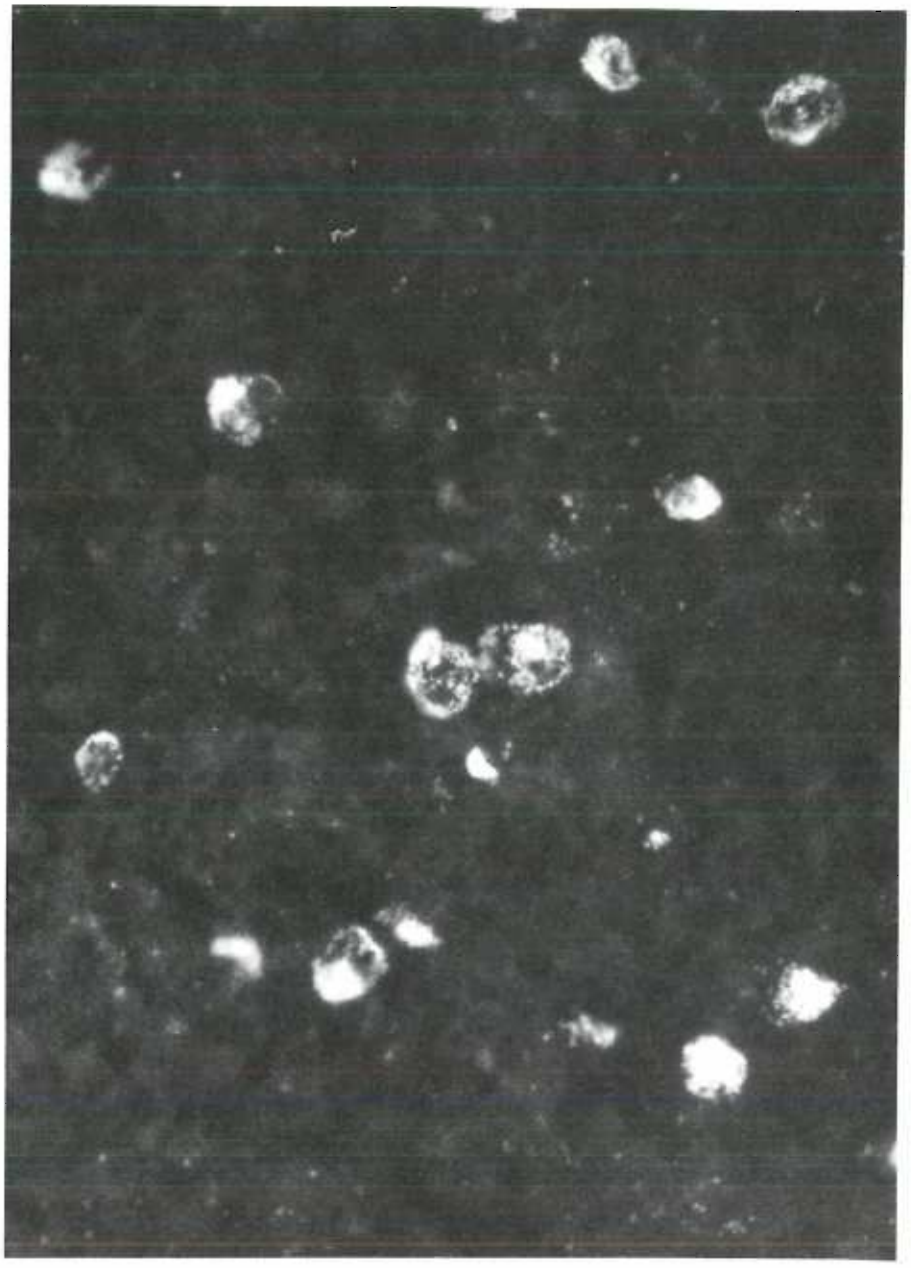


Figure 3.

Impression Smear of Lymph Node from Patient #30 Stained with Measles Virus Antiserum (1660X).



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Appendix A.

Mumps Virus Fluorescent Focus Forming Assay.

Research and clinical evaluation of mumps virus have been hindered in recent years due in part to the lack of a rapid and efficient method of quantitation that could be used for different strains of mumps virus.

A number of assays have been described for myxoviruses (6), mumps virus (2,4,5), and other paramyxoviruses (1,3,7,8,9). These have included hemagglutination, plaque formation, non-cytopathic hemadsorption plaques, and immunoperoxidase staining. However, the methods reported to date have either limited applicability for mumps virus, due to the strain specificity or lack the rapidity which the fluorescent antibody technique offers for the quantitation of mumps virus.

Fluorescent assay techniques for several viruses have been previously reported. A fluorescent focus assay for adenoviruses 1, 2 and 12, adeno-associated virus type 1 and reovirus type 1 used human amnion cells (10).

Due to the lack of a rapid and efficient method of assaying mumps virus, a 48 hour indirect fluorescent antibody technique for the quantitation of mumps virus was developed using HeLa cell monolayers. A dose-response curve illustrating the dose dependency of the assay and a description of the assay is contained in Paper 1 of this thesis. The assay worked equally well

with the Enders or Jones strains of mumps virus (Table 1) or a clinical mumps virus isolate obtained from the Center for Disease Control (Atlanta, Ga.). The reproducibility of the assay was demonstrated by several experiments using different virus samples (see Table 2). Fluorescing foci did not appear when attempts were made to stain with fluoresceinated Newcastle disease virus antiserum or measles virus antiserum. The activity of the mumps virus antiserum, specific for mumps virus, could be removed by absorbing with purified mumps virus or cells persistently infected with mumps virus. The mumps-specific activity could not be removed using uninfected BHK, HeLa, or CEF cells. Examples of fluorescing foci of infection can be seen in Figure 1 of this appendix, and Figure 2 of Paper I of this thesis.

Table 1. Assay of Jones and Enders strains of mumps virus.

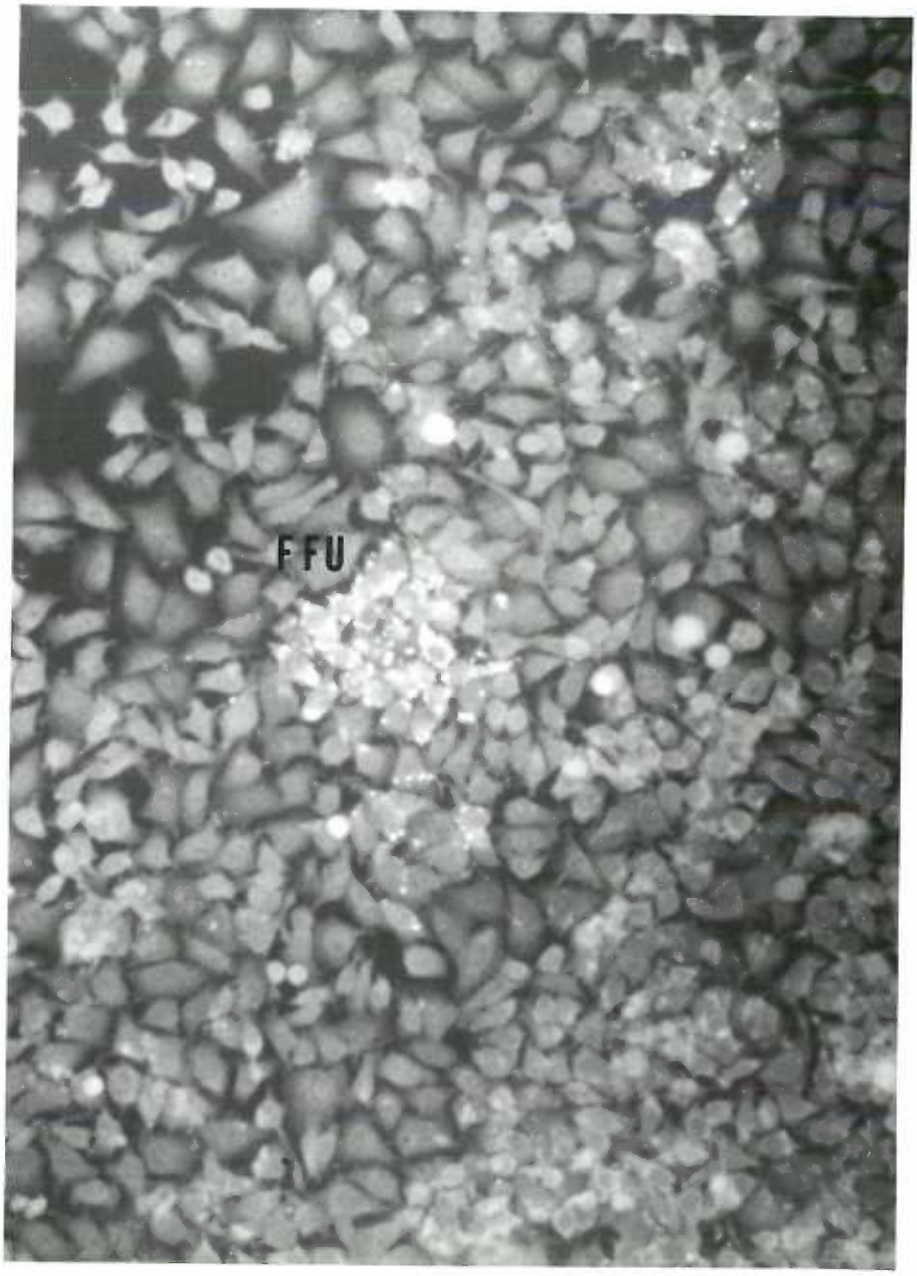
Strain	Calculated titer
Jones	7.92×10^2 FFU/ml
Enders	1.55×10^4 FFU/ml

Table 2. Reproducibility of mumps virus fluorescent focus forming assay.

Mumps sample	Calculated number of fluorescent foci/ml	% variation of mean
A1	7.55×10^5	1
A2	7.70×10^5	1
Mean	7.63×10^5	
B1	1.12×10^7	2.6
B2	1.20×10^7	4.4
B3	1.16×10^7	0.9
B4	1.12×10^7	2.6
Mean	1.15×10^7	

Figure 1.

Mumps Virus Focus Forming Units (FFU) in HeLa Cell Monolayers. Magnification is 420X.


FFU

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Appendix B.

Immunofluorescence in BHKpi Cultures.

In addition to the cytoplasmic fluorescence observed in BHKpi cultures, nuclear immunofluorescence could also be observed. This is in contrast to the exclusive cytoplasmic fluorescence observed in lytic infections with mumps virus (2,7). However, hamster adapted strains of mumps virus have been found to produce intranuclear inclusions in the uterus and placenta of the pregnant hamster (5). The nuclear fluorescence in the BHKpi system appeared within one to two days after subculturing and decreased with time in culture, but never disappeared entirely. Cytoplasmic immunofluorescence appeared to increase with time in subculture. The nuclear fluorescence appeared to be nucleolar in nature and suggests the possibility of a nuclear maturation which is associated with the nucleolus during the course of the persistence as suggested by Bukrinskaya (1) for Sendai virus. Nuclear involvement with paramyxoviruses has been previously documented for NDV (4), measles (6), and mumps (5). Furthermore, East and Kingsbury found that actinomycin D severely inhibited mumps virus replication and 50S RNA accumulation (3). It was therefore suggested that continued transcription of the host cell genome is involved in mumps virus genome replication and virion production.

Figure 1 is a photomicrograph of control BHK cells.

No specific immunofluorescence could be observed at any time indicative of mumps virus, and control BHK cells were used as negative controls in all experiments utilizing BHKpi immunofluorescence. In addition, BHK cells showed no specific fluorescence when attempts were made to stain with either fluoresceinated measles antiserum or Newcastle disease virus antisera. BHK cells, however, demonstrated a very small amount of autofluorescence and a degree of nonspecific generalized fluorescence which could be totally removed by counterstaining with Evans blue.

Figures 2 and 3 are photomicrographs of BHKpi cells stained at d1 and d5 post-subculture respectively. The nuclear fluorescence, observed shortly after subculture, appeared nucleolar-like with approximately 4 to 10 fluorescing sites and up to 50 fluorescing sites in the larger syncytia. The cytoplasmic fluorescence usually seen during the course of subculture was granular in nature, characteristic of a lytic mumps virus infection. There appeared to be a progression of mumps-specific immunofluorescence from the nucleus to the cytoplaam during the course of subculture. The details, procedures and controls for this reaction are discussed in Paper 1 of this thesis.

The nature of the nuclear involvement observed in the BHKpi persistence is to date unexplained. Experiments from this laboratory using wild-type mumps virus (either Enders, Jones or clinical mumps virus strains) have failed

to show any evidence of a nuclear involvement either very early after infection or very late. All of the immunofluorescence observed during the course of a lytic infection was cytoplasmic. This is in contrast to reported observations with NDV in which a nuclear stage was observed very early after infection.

Furthermore, experiments using MuVpi have also failed to demonstrate any nuclear stage during the course of a lytic infection. Therefore, the nuclear involvement observed in the BHKpi persistence appears to be a result of the virus-cell interaction.

Figure 1.

Control BHK Cells Stained with Mumps Virus Antiserum (1660X).

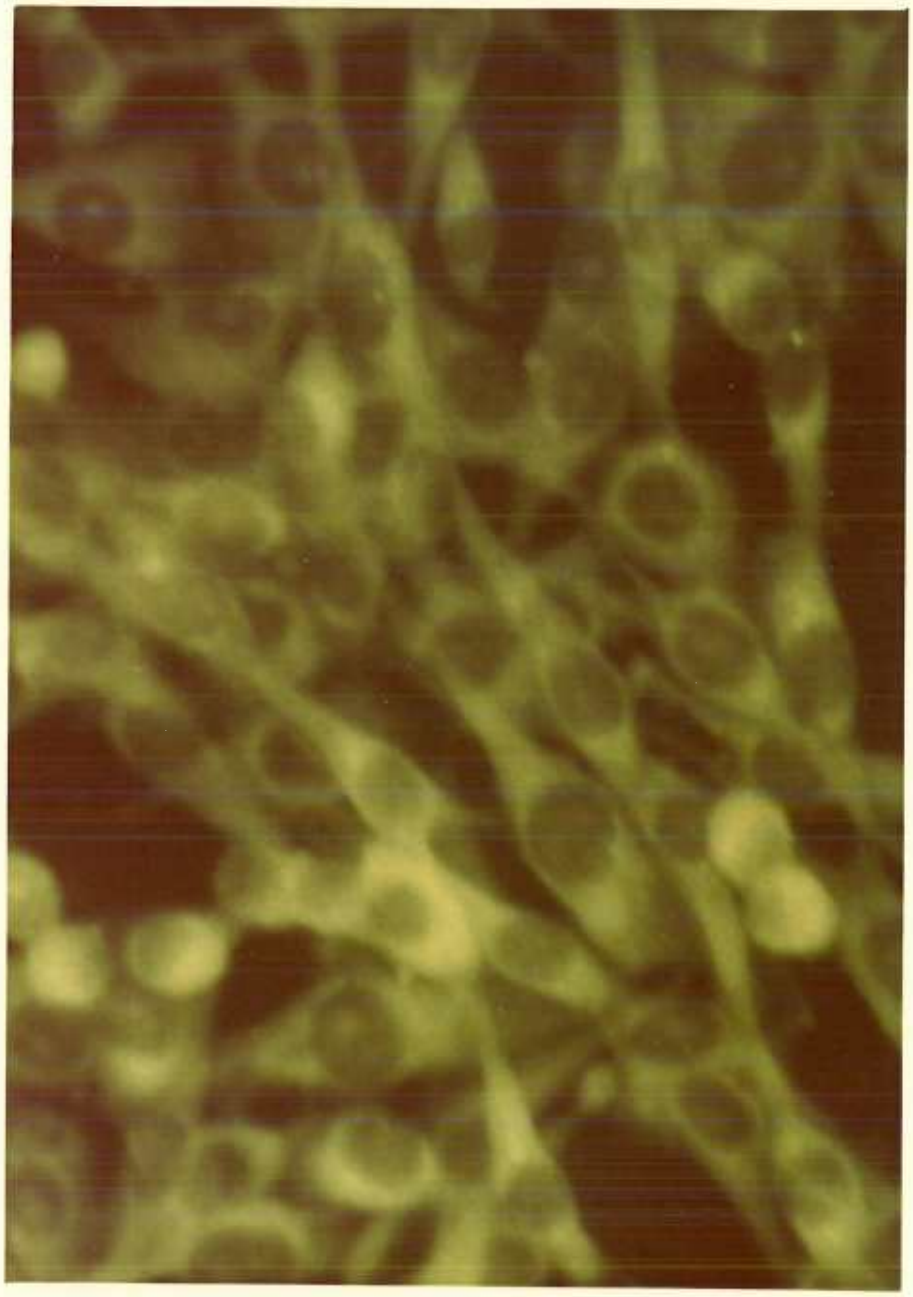


Figure 2.

BHKpi Cells Stained with Mumps Virus Antiserum at Day 1 Post-Subculture.

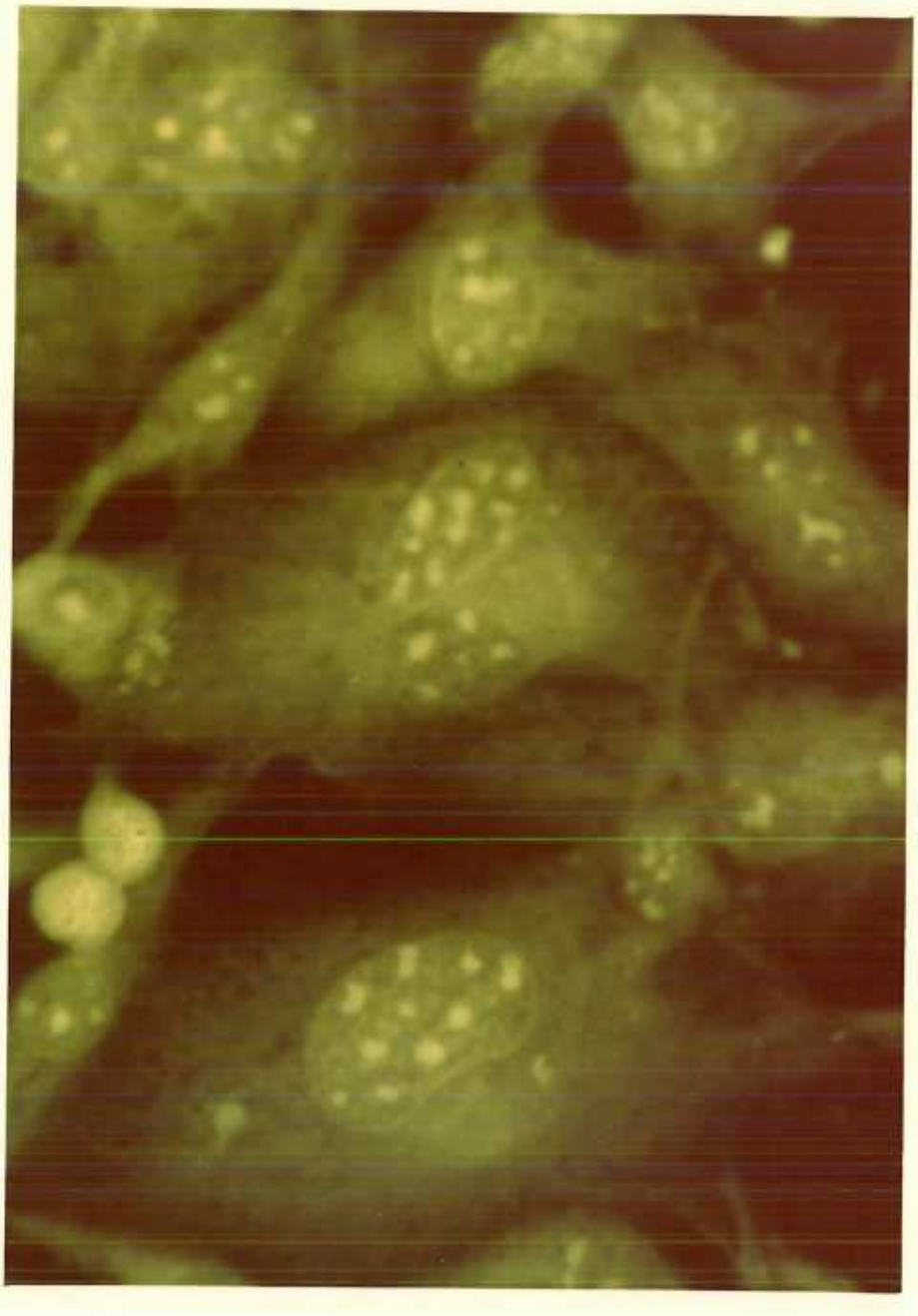
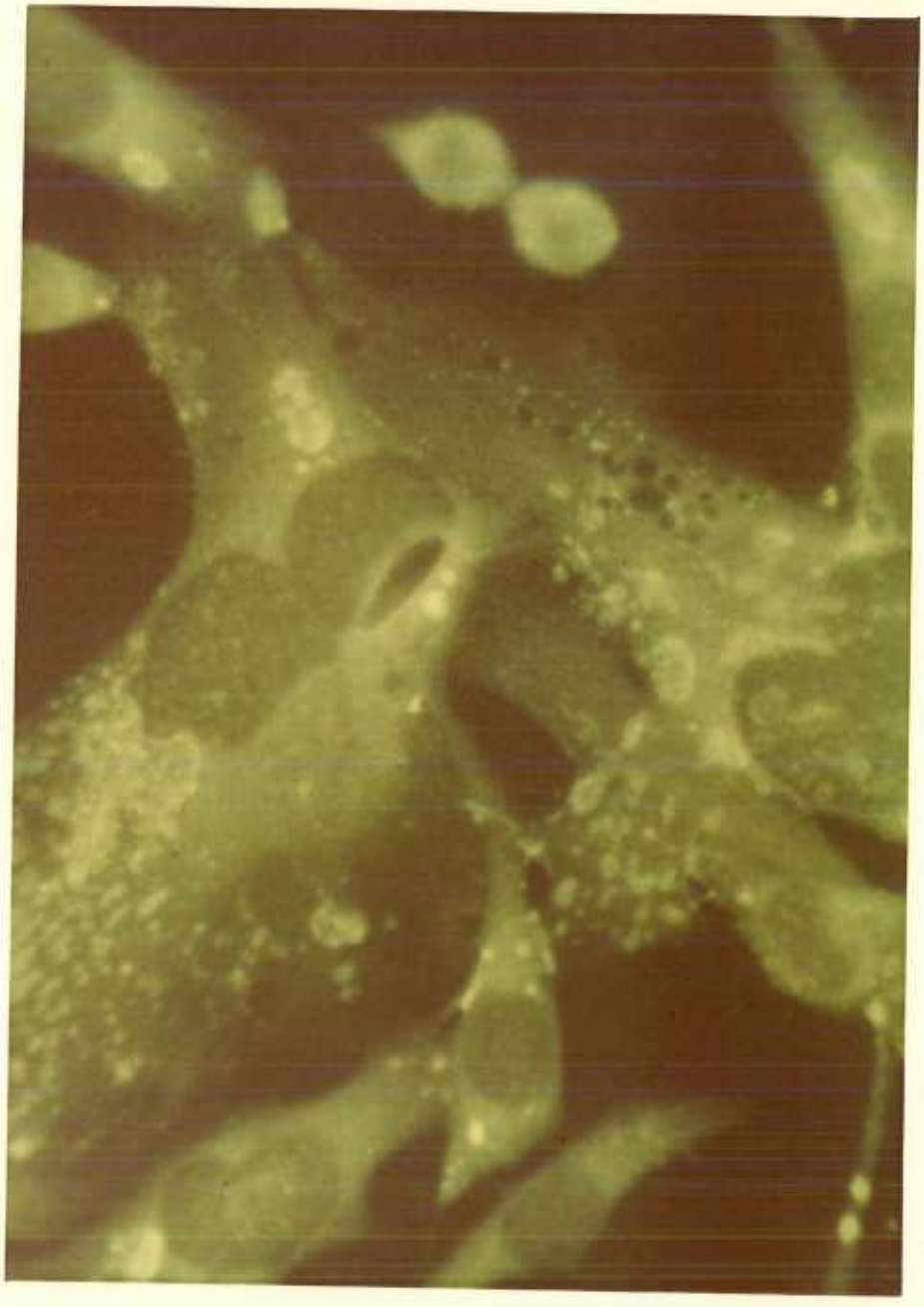


Figure 3.

BHKpi Cells Stained with Mumps Virus Antiserum at Day 5 Post-Subculture.



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Appendix C.

Electron Microscopy of BHKpi Cultures and Mumps Virus.

BHK and BHKpi cultures were analyzed electron microscopically to determine the nature of the nuclear and cytoplasmic immunofluorescence (see Figures 1-3). The ultrastructure of the BHK-21 cells was similar to that found by Soto and Castejon (1). Electron micrographs of the BHKpi cultures revealed the presence of microtubules in the cytoplasm of the cells. The tubules ranged in diameter from 180-190 Å, characteristic of paramyxovirus nucleocapsids. Intranuclear bodies also seemed to be present in the BHKpi cells with occasional microtubules measuring 125 Å in diameter, slightly smaller than those present in the cytoplasm. Control BHK cells did not contain these intranuclear bodies. The nature of the structures is being further investigated.

The procedures used for electron microscopy of BHK and BHKpi cells were briefly as follows. Cell monolayer cultures were rinsed twice with CMF-PBS and fixed <u>in situ</u> for 1 h at room temperature with 1.4% glutaraldehyde and 1.5% formaldehyde in CMF-PBS. The cells were removed mechanically from the flask with a rubber policeman and centrifuged at 400 x g for 15 min at 4 C. The pellet was resuspended in CMF-PBS and centrifuged again. The washed cells were post-fixed in 1% (w/v) osmium tetroxide in

CMF-PBS for 1 h at room temperature. The cells were then washed twice in CMF-PBS before dehydration in a graded series of ethanol (75%-95%). Following dehydration, the cells were embedded in Epon 812 which was allowed to polymerize overnight at 60 C. Sections were cut and examined with a Philips EM-200 electron microscope.

MuVo and MuVpi samples were purified on sucrose gradients (15-65%) and resuspended in 0.02 M NH_3COOH buffer. A drop of this material was used to float grids, which were then stained with 1% ammonium molybdate solution. Specimens were then air dried in a dust-free environment and observed on a Philips EM-200 electron microscope (see Figures 6 and 7).

The electron microscopic studies were performed in collaboration with Robert Brooks of the Department of Pathology and John Huntington of the Department of Microbiology and Immunology, UOHSC.

Electron micrographs of MuVo are contained in Figures 4 and 5. The virions were observed to be very pleomorphic, typical of paramyxoviruses and ranged in diameter from 100 to 300 nm in diameter. MuVo and MuVpi appeared identical electron microscopically.

Figure 1.

Electron micrograph of control BHK-21 cell. Bar represents 500 nm.

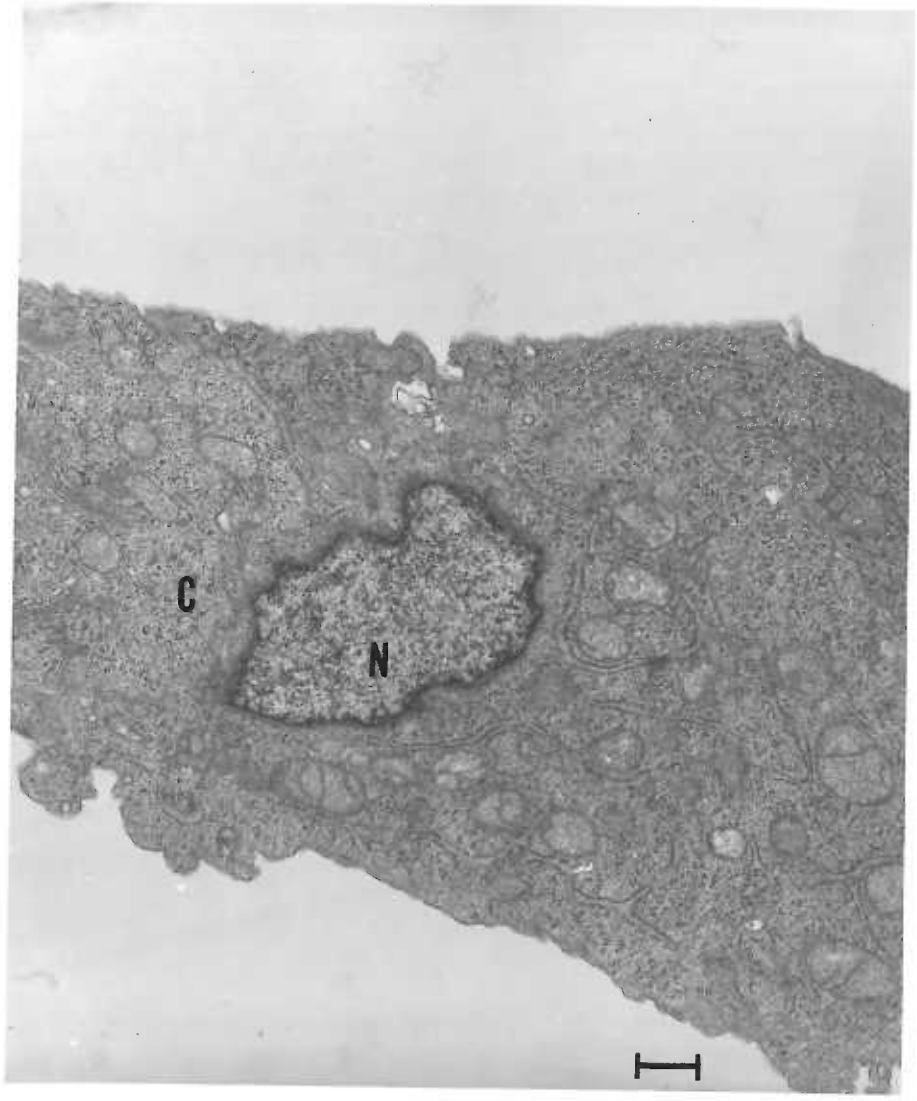

C
N

Figure 2.

Electron micrograph of BHKpi cell. Bar represents 500 nm.

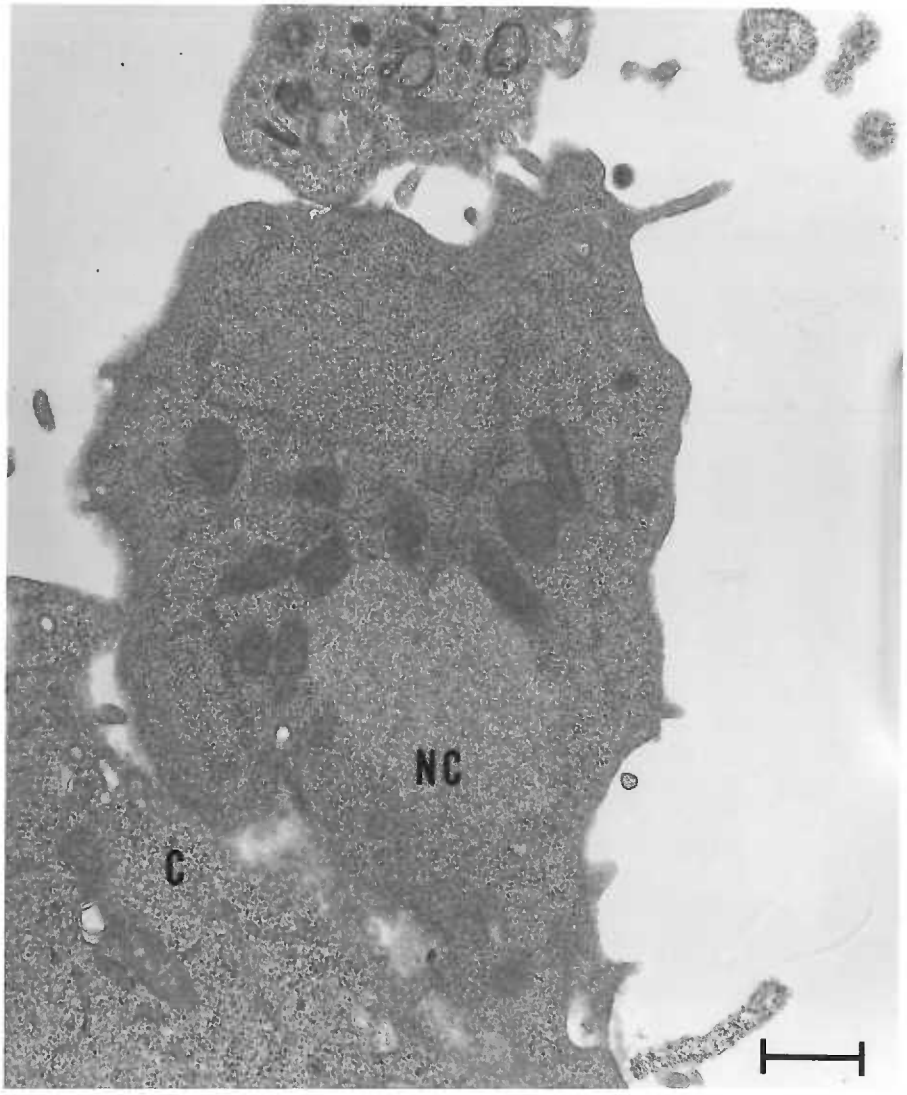

NC
C

Figure 3.

Electron micrograph of BHKpi cell. Bar represents 500 nm.

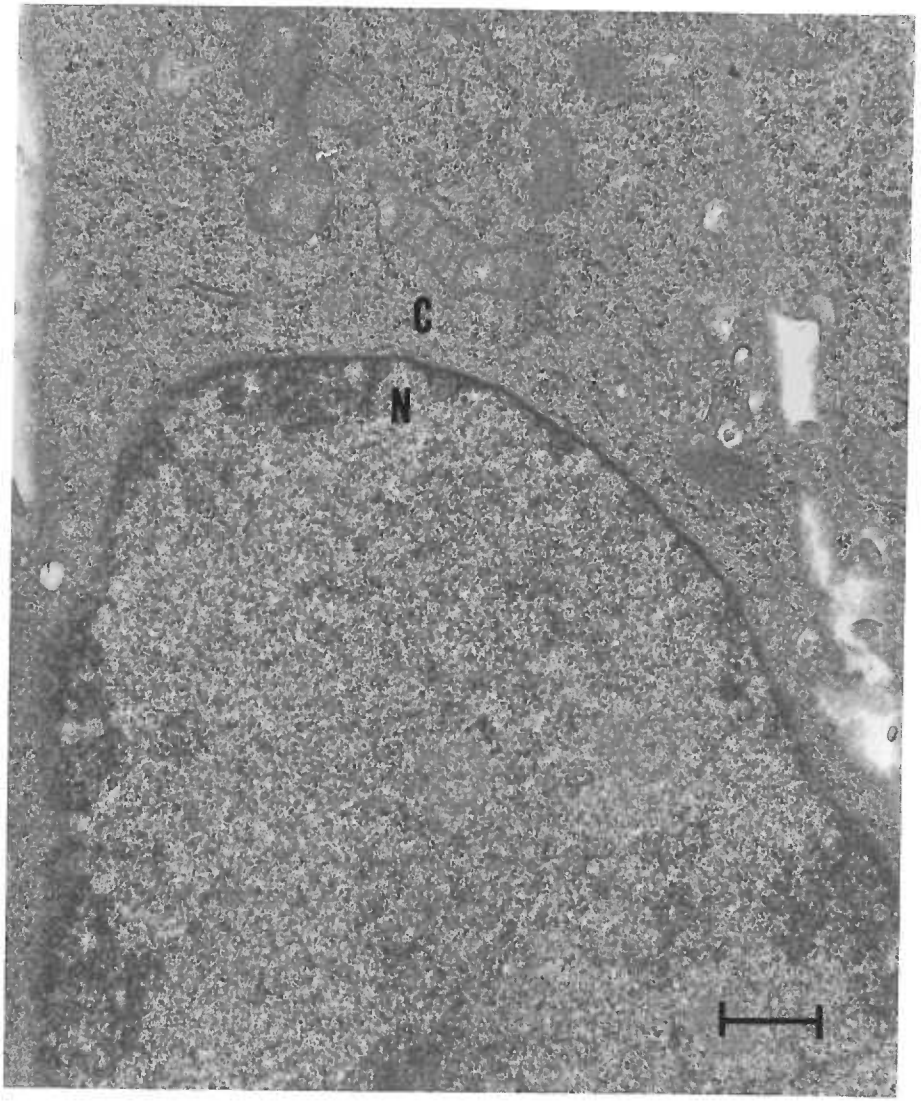

C
N

Figure 4.

Electron micrograph of sucrose gradient-purified MuVo. Bar represents 500 nm.

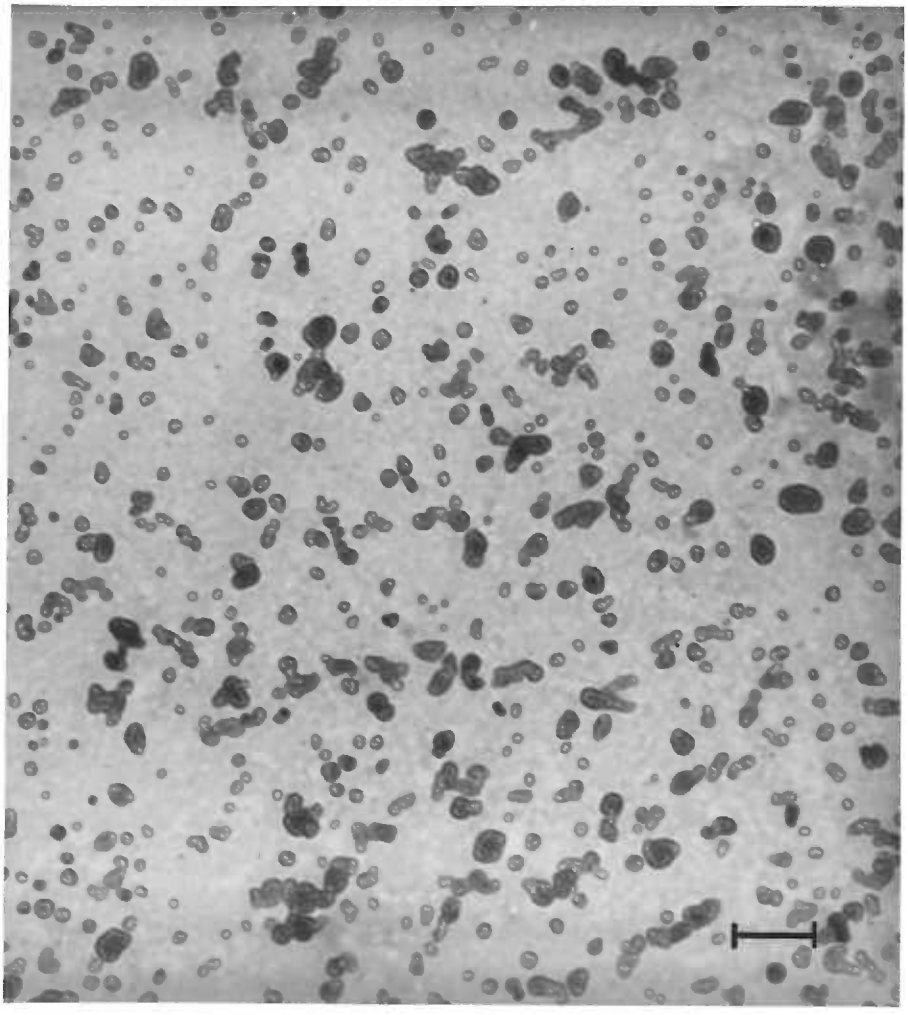
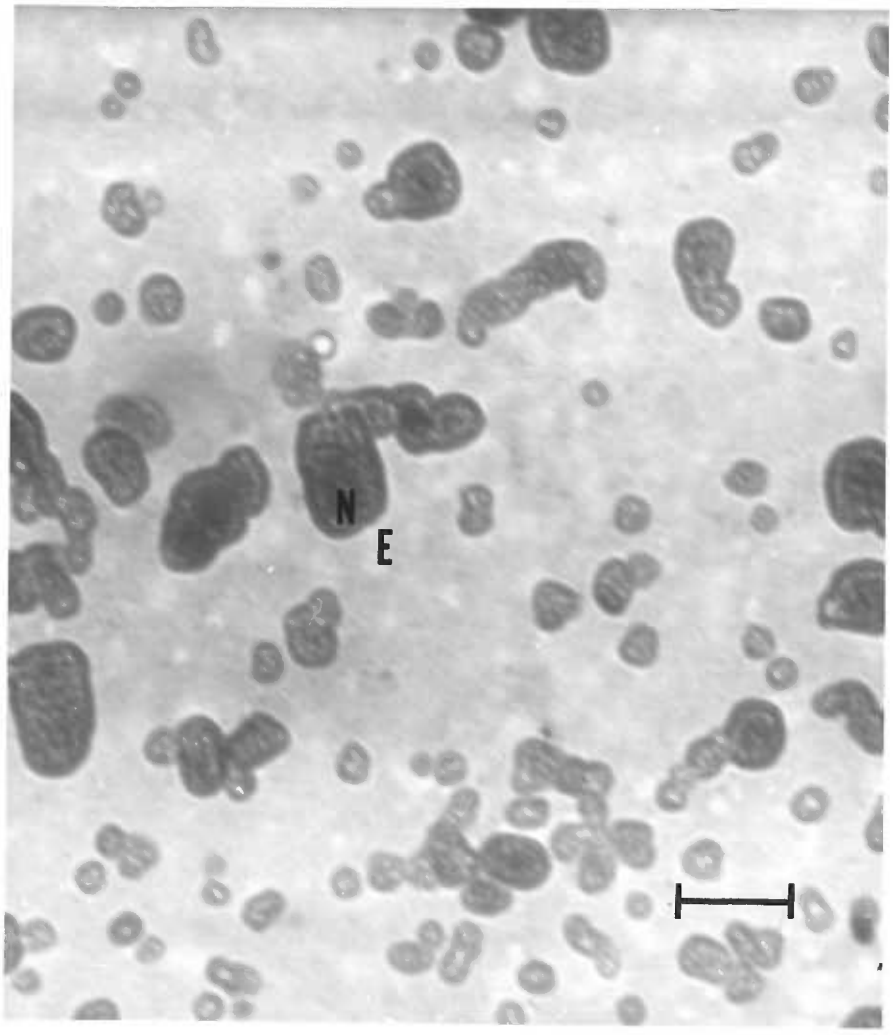


Figure 5.

Electron micrograph of sucrose gradient-purified MuVo. Bar represents 250 nm.


N
E

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Appendix D.

Sucrose Gradient Analysis of MuVo and MuVpi.

MuVo and MuVpi were analyzed by sucrose gradient centrifugation to determine if defective virus particles could be observed as a distinct peak by radioactivity. Samples of MuVo were amplified in BHK cell cultures and labeled with ^{3}H-Uridine (50 μCi/ml), and BHKpi cultures were grown in the presence of ^{3}H-U. Virus populations were clarified (8,000 RPM, 30 min, Sorvall RC-2, GSA rotor), pelleted (23 K RPM, 2 h, Beckman L5-65, type 30 rotor), and purified on a 15-65% sucrose-D_2O gradient (39 K RPM, 4 h, Beckman L5-65, SW-41 rotor).

Typical profiles can be seen in Figures 1 and 2. The linearity of each gradient was determined by direct weight measurement in addition to Abbe refractometer readings. MuVo migrated as a single band and the density was observed to be 1.19 gm/cc, typical of mumps virus. MuVpi migrated identical to that of wild-type mumps virus with a mean density of 1.19 gm/cc. No significant radioactive peaks were observed in addition to the main virus band. However, this does not rule out the presence of defective particles since these could be present in the main virus band.

Figure 1.

Sucrose gradient analysis of ^{3}H-U labeled MuVo (15-65%).

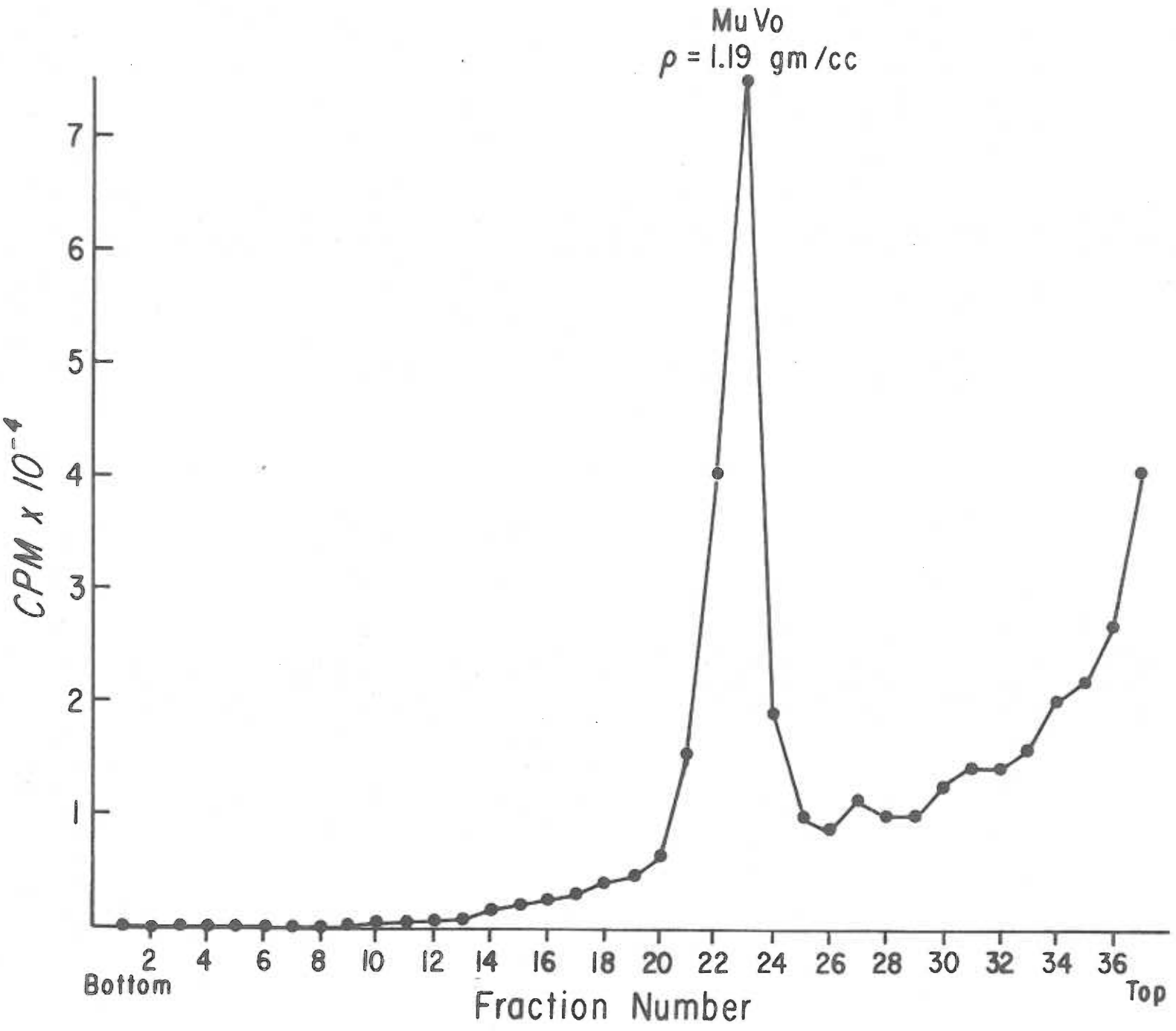
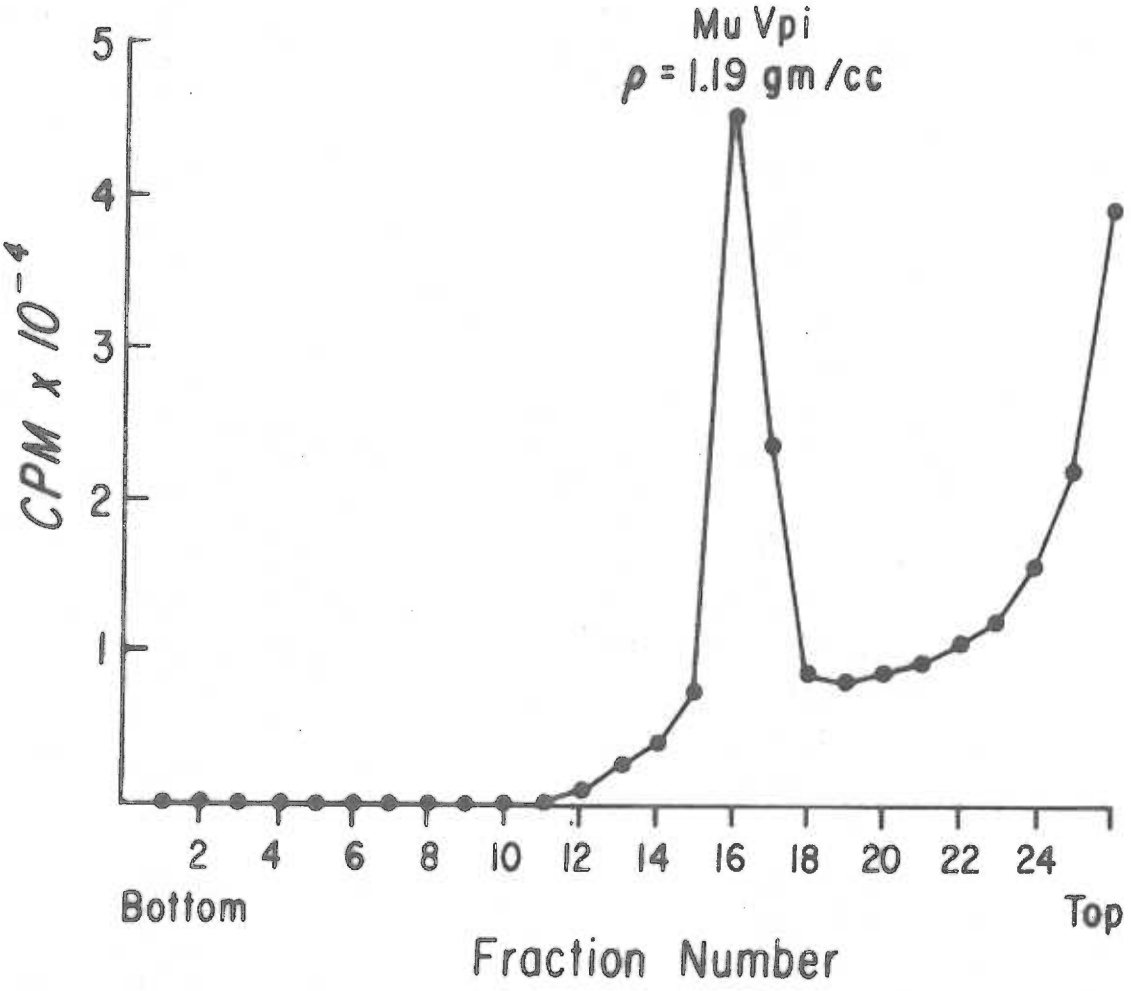

Mu Vo
ρ = 1.19 gm/cc
CPM x 10⁻⁴
7
6
5
4
3
2
1
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
Bottom
Top
Fraction Number

Figure 2.

Sucrose gradient analysis of ^{3}H-U labeled MuVpi (15-65%).


Mu Vpi
ρ = 1.19 gm/cc
CPM x 10⁻⁴
5
4
3
2
1
2
4
6
8
10
12
14
16
18
20
22
24
Bottom
Top
Fraction Number

Appendix E.

Transfection with BHKpi and BHK DNA.

Zhdanov and Parfanovich (8) have reported that measles virus DNA transcripts are integrated into the cell genome of chronically infected chicken embryo fibroblasts. In addition, Simpson and Iinuma (7) have recovered infectious DNA from Hep-2 cells latently infected with respiratory syncytial virus.

To determine if mumps virus RNA copies had been transcribed into DNA, transfection studies were performed in HeLa cells with purified DNA from BHK and BHKpi cells.

BHK and BHKpi cells were grown and scraped from monolayer cultures (1×10^8 cells). The cells were resuspended in 1X SSC (standard sodium citrate) and 0.5% SDS along with 50 μg/ml of self-digested pronase (8 h at 37 C), incubated at 37 C for 1 h, then extracted 3 times at room temperature with H_2O (sterile distilled) saturated phenol. This was followed by extraction with chloroform-isoamyl alcohol (24:1). Nucleic acid was precipitated with two volumes of cold (5 C) absolute ethanol and the nucleic acid was collected on a glass stirring rod, air dried, and redissolved in 1X SSC. The remaining nucleic acid was centrifuged at 10,000 RPM (HB-4 Beckman rotor) for 30 min, air dried, and redissolved in 1X SSC. Pancreatic RNase (boiled for 10 min) was then added to give

a final concentration of 50 μg/ml and allowed to incubate at 37 C for 1 hr. The mixture was then extracted 1X with phenol and 2X with chloroform-isoamyl alcohol (24:1), spooled and redissolved in 1 ml of 1X SSC. The 260/280 ratios were determined on an aliquot of the BHK and BHKpi cell DNA and are contained in Table 1. Cultures of BHK cells were incubated at 37 C, and cultures of BHKpi were incubated at 33, 37 and 39 C. The 260/280 ratios were higher (i.e. containing less contaminating protein) than those used by Temin and colleagues (3,6) who isolated infectious reticuloendotheliosis virus DNA from infected cells. The total amount of DNA was determined by optical density (A_{260}) with 1.0 O.D. unit = 50 μg double-stranded DNA.

HeLa cell monolayers were transfected by the method of Boyd and Butel (2) and Al-Moslik and Dubes (1). Briefly, monolayer cell cultures were grown in 10 x 35 mm plastic tissue culture dishes to confluency. The media was removed and rinsed with calcium and magnesium free phosphate buffered saline (CMF-PBS) for 15 min. The cells were then sensitized with diethylaminoethyl (DEAE) dextran (1 mg/ml; 0.25 ml) for 9 min at 23 C. The cells were then rinsed 3 times with CMF-PBS. DNA was then added in concentration of 50 or 100 μg/plate (0.25 ml) in duplicate and allowed to incubate at room temperature for 15 min. DNA was tested from BHKpi cultures grown at 33, 37 or 39 C and from BHK

cultures grown at 37 C. The cell cultures were then rinsed with CMF-PBS and 2 ml of maintenance media was added to each plate and incubated at 33, 37 or 39 C for each DNA sample under examination, in a 5% CO_2 atmosphere. BHK and BHKpi cell DNA (100 μg) incubated (37 C for 1 hr) with 100 μg/ml of DNase (Worthington Biochemical Co.) in 0.01 M Tris, 0.01 M NaCl, 0.001 M Mn^{++} buffer, DEAE alone as well as non-transfected cells served as controls. The cells were monitored for the presence of viral antigens by hemadsorption, immunofluorescence, and for infectious virus. Neither viral antigens nor infectious virus was detected or recovered at 24 hr, 48 hr, or 7 days after DNA adsorption.

Transfection experiments were carried out several times using the above protocol and in addition a $CaCl_2$ sensitization protocol as per Graham and Van Der Eb (4,5), all producing the same results. However, it must be stressed that these results, although negative, do not rule out the possibility that infectious DNA may be present in BHKpi cells. The sensitivity of molecular hybridizations would more conclusively demonstrate the presence or absence of mumps-specific infectious DNA in BHKpi cells.

Table 1. 260/280 ratios of BHK and BHKpi DNA.

		260	280	260/280
BHK	(37 C)[1]	0.404	0.208	1.94
BHKpi	(33 C)	1.400	0.670	2.09
BHKpi	(37 C)	1.705	0.935	1.82
BHKpi	(39 C)	1.590	0.840	1.89

[1] Temperature at which cells were grown.

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Appendix F.

Lymphycyte Transformation in Hodgkin's Disease and Non-Hodgkin's Disease Patients.

In preliminary studies, we have detected mumps virus antigens by indirect immunofluorescence in lymph node biopsies from 12 patients with Hodgkin's disease (HD) (see Paper II of this thesis). Careful adsorption and blocking experiments confirmed that the fluorescence was specific for mumps virus antigens and that these observations were unique to HD. The persistence of mumps virus in the lymphoid tissue of these patients could be explained by a deficiency in immune responsiveness to this agent. Since cell-mediated immunity (CMI) is considered crucial to viral resistance, immune responsiveness was assessed by lymphocyte transformation to mumps virus in both Hodgkin's and non-Hodgkin's lymphoma (DHL) patients. Immune lymphocytes are thought to interact with viral antigens and produce interferon (an antiviral lymphokine) which prevents host cells from allowing viral replication.

Lymphocyte cultures from HD and NHL patients were challenged <u>in vitro</u> with phytohemagglutinin (a nonspecific mitogen) and specific viral antigens.

Lymphocyte transformation was measured by tritiated thymidine uptake in a modified whole blood culture system. A total of 2.5 ml of heparinized blood (60 u /ml) was diluted to 50 ml with medium RPMI 1640 containing antibiotics

and 25 millimoles Hepes buffer. Two tenths microgram of phytohemagglutinin-P (PHA), or 0.1 ml of standard strength Herpes, CMV, measles, or mumps antigens were added to duplicate 3 ml cultures. This dilution of blood consistently supplied 800,000 to 1,000,000 lymphocytes per culture. The cultures were incubated for five days at 37 C with 1 microcurie of tritiated thymidine (specific activity, 6.8 millicuries/millimole) added 24 hr prior to the end of the incubation period. The cultures were harvested on glass-fiber filters and washed with 3% acetic acid. The filters were placed in 20 ml vials with a 10 ml scintillation "cocktail" and counted in a liquid scintillation counter.

The data (Table 1) suggest that the HD patients may have a selective deficiency in mumps virus responsiveness. In general, the patients with HD responded poorly to mumps virus when compared to NHL patients or normal controls (14/18 controls showed significant stimulation). In the HD group 4/20 demonstrated significant responsiveness and and in the NHL group 10/14 showed significant responsiveness. It appeared that of the patients tested there appeared to be no significant pattern for responsiveness to cytomegalovirus, Herpes, or measles viruses. It should be pointed out that this is preliminary data and must be extended to other patients correlating with stage, histopathologic grouping, previous therapy, and confirmed with

additional immune measurements before firm conclusions can be drawn.

One of the important aspects of this finding, if indeed Hodgkin's patients do possess an anergy to mumps virus, is that reconstitution of the T-cell arm of the immune response may be possible with the use of mumps-immune transfer factor. To date only familial transfer factor has been used in cases of Hodgkin's disease with very limited success.

Table 1. Immune responsiveness in patients with Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL).

Lymphocyte transformation (CPM x 10^{-3} of ^{3}H-thymidine uptake) in cultures stimulated with PHA or mumps virus.**

Patients with HD			Patients with NHL		
Reference No.	PHA	Mumps	Reference No.	PHA	Mumps
11	8.0	0.1	31	58.6	0
12	24.5	0	32	74.0	2.6*
13	36.1	3.5*	33	74.4	2.3*
14	13.6	0.4	34	96.5	2.2*
15	5.6	0	35	77.1	1.5*
16	3.7	0	36	74.0	2.1*
17	47.2	0	37	31.0	1.8*
18	57.3	0.3	38	19.2	1.3*
19	32.4	0.7	39	69.3	4.7*
20	46.4	1.1*	40	9.1	0.4
21	160.0	6.2*	41	41.7	0.1
22	34.0	0	42	15.6	9.6*
23	2.7	0	43	129.0	0.5
24	24.6	0.6	44	27.0	1.1*
25	40.6	0.6			
26	20.0	0			
27	10.1	0			
28	34.5	0.1			
29	44.5	0.2			
30	76.0	1.4*			
*Positive Responders		4/20			10/14

**Values were corrected for background levels; Positive responders represented a minimum of a 2-fold reaction above background.

Appendix G.

BHKpi Temperature Up-Shift and Antiserum Cure Experiments.

BHKpi cultures were shifted from 37 C to 39 C to determine if an increase in temperature would cure the infection of mumps virus involvement. BHKpi p35 at cell confluency was shifted from 37 to 39 C. Control cells were kept at 37 C for comparison; these cells demonstrated 5-10% Had, 70-90% mumps immunofluorescence, and approximately 10^2 FFU/ml released mumps virus.

The results of temperature shift of BHKpi cultures are shown in Table 1. A decrease of both hemadsorption and immunofluorescence were observed; however, the cultures were not cured of mumps virus persistence. The released virus of these cultures was very low, approximately 10 FFU/ml.

BHKpi cultures were also subcultured into mumps virus antiserum in an attempt to determine if the persistence could be cured of viral antigens by the presence of mumps-specific antiserum. The mumps antiserum (Flow Laboratories, Lot G828016) was titered and found to contain 256 neutralizing units per 0.5 ml. A 1:256 dilution totally neutralized 2.0×10^3 FFU of mumps virus. Therefore a 1:200 dilution of antiserum was chosen for the antiserum cure experiment since the mumps persistence released approximately 10^2 FFU/ml. The results of this experiment are contained in Table 2. No released virus could be detected at any passage. The control cells were identical to the controls

used in the temperature up-shift experiment. It appeared that a decrease in mumps antigen expression was observed; however, the persistence was not cured. When cultures were shifted back to media lacking mumps antiserum, the percentage of mumps antigen appeared to increase slightly with respect to hemadsorption. At cell passage 1 the BHKpi cultures had approximately 5% Had, at passage 2, 5% and at passage 3, 20% Had.

In an attempt to determine if normal guinea pig serum exhibited the same effect as mumps antiserum, and what effect higher and lower concentrations of mumps antiserum would have on the persistence, the following experiment was carried out. BHKpi cultures were subcultured into 1:40, 1:200 and 1:1000 dilutions of normal guinea pig serum and 1:40, 1:200 and 1:1000 dilutions of mumps virus antiserum. All cultures were followed by hemadsorption. Control BHKpi cells were maintained without antiserum and produced 30-70% Had, and 10^2 FFU/ml released virus during the course of the experiment. The results of this experiment are contained in Table 3, and indicate that normal guinea pig serum had no significant effect on Had of the persistence. The percentage of Had did appear to be influenced by the concentration of mumps antiserum in the medium; however, the persistence was not cured with a 1:40 dilution of antiserum through cell passage 5.

Table 1. Hemadsorption and immunofluorescence of BHKpi cultures at 39 C.

	% Hemadsorption	% Immunofluorescence
BHKpi p1*	1-2	10-20
BHKpi p2	1-2	1-2
BHKpi p3	1-2	50-60

*Temperature up-shift of BHKpi p35 from 37 to 39 C was designated cell passage 1.

Table 2. Hemadsorption and immunofluorescence of BHKpi cultures in 1:200 mumps antiserum.

	% Hemadsorption	% Immunofluorescence
BHKpi p3	0.5 - 1	50
BHKpi p4	0.5 - 1	50
BHKpi p5	5.0	50

Table 3. Hemadsorption of BHKpi cultures in 1:40, 1:200 and 1:1000 dilution of normal guinea pig serum or mumps virus antiserum.

Cell Passage	Serum		% Hemadsorption
p1	NGP*	1:40	60
p1	NGP	1:200	60
p1	NGP	1:1000	60
p1	MuAs*	1:40	20
p1	MuAs	1:200	50
p1	MuAs	1:1000	50
p2	NGP	1:40	60
p2	NGP	1:200	60
p2	NGP	1:1000	60
p2	MuAs	1:40	20
p2	MuAs	1:200	40
p2	MuAs	1:1000	50
p3	NGP	1:40	70
p3	NGP	1:200	70
p3	NGP	1:1000	70
p3	MuAs	1:40	ND**
p3	MuAs	1:200	30
p3	MuAs	1:1000	50
p4	NGP	1:40	60
p4	NGP	1:200	60
p4	NGP	1:1000	60
p4	MuAs	1:40	20
p4	MuAs	1:200	20
p4	MuAs	1:1000	40
p5	NGP	1:40	70
p5	NGP	1:200	70
p5	NGP	1:1000	70
p5	MuAs	1:40	5
p5	MuAs	1:200	20
p5	MuAs	1:1000	40

*NGP, Normal Guinea Pig serum; MuAs, Mumps Virus Antiserum;
**ND, Not Determined

Appendix H.

Summary: A Hypothesis for the Mechanism of Maintenance of the Persistent Infection

The results presented in this dissertation imply that temperature-sensitive mutants of mumps virus may play an integral role in both the establishment and maintenance of the carrier state *in vitro*. The persistently infected BHK cells have been shown to produce infectious, temperature-sensitive virus which is much less cytopathic than wild-type mumps virus and which readily establishes a persistent infection.

Several mechanisms for establishment and maintenance of persistent viral infections *in vitro* include interferon, antibody, genetically resistant cells, temperature-sensitive or other viral mutants, defective interfering particles and DNA intermediates, none of which are mutually exclusive.

In the BHKpi system, no interferon could be detected, the infection was established and maintained in the absence of mumps virus antibody and no evidence for genetically resistant cells was observed. No defective virus particles were observed in the BHKpi persistence. Sucrose gradient centrifugation of ^{3}H-uridine labeled MuVpi demonstrated a single virus band migrating at a density of 1.19 gm/cc, characteristic of mumps virus. Therefore, if defective virus particles are present they are contained in the major

virus band. This, then, does not rule out defective virus particles as a possible contributing factor in the mechanism of maintenance for this infection. The possible involvement of a DNA intermediate in BHKpi cells has also been investigated. However, no evidence for DNA intermediates has been found in BHK cells persistently infected with mumps virus. Transfection studies utilizing DNA isolated from BHKpi cells (grown at 33, 37 and 39 C) did not demonstrate hemadsorption, immunofluorescence or released virus associated with mumps virus (when adsorbed to DEAE-sensitized HeLa cell monolayers). However, it must be stressed that these results, although negative, do not rule out the possibility that infectious DNA may be present in BHKpi cells. The sensitivity of molecular hybridizations would more conclusively demonstrate the presence or absence of mumps-specific infectious DNA in BHKpi cells.

The temperature-sensitivity of the virus produced from the persistence, then, appears to be the most apparent change which the virus has undergone during the course of the persistence. In addition, the cells which are persistently infected show a nuclear involvement not observed in wild-type infections with mumps virus. The nuclear stage may be involved with maturation of the virions since there appears to be a shift from the nucleus to the cytoplasm during subculture of the BHKpi.

The exact nature of the temperature effect is being

further investigated. It appears that the effect is not due merely to attachment or adsorption of the viruses since shifting from 39 to 33 C 1 hour after virus adsorption does not affect the infectivity.

It appears that at least two mechanisms may be functioning to maintain this persistent infection. We know that temperature-sensitive virus is selected for very early in the infection, most probably from pre-existing spontaneous ts mutants. Although the virus released from the infection is relatively non-cytopathic, it appears to be lethal to the host cells since no virus-producing clones could be isolated. Therefore one possible mechanism could be of an equilibrium type. That is, producer cells can release low levels of infectious virus which reinfects normal uninfected cells. The cell killing by this virus must approximately equal the rate of growth of the susceptible normal cells.

A second, concurrent mechanism must be proposed to account for the fact that infected clones isolated from the persistent infection do not produce virus. These clones express mumps virus antigens which do not dilute out upon cell passage and are resistant to superinfection with homologous virus. In addition recent experiments have demonstrated that infectious virus can be rescued from these clones by a temperature down-shift from 37 to 33 C. A possible explanation for

these observations is that there is a very fine balance between the amount of infectious virus being produced and defective interfering particles. In this way very little infectious virus would be released. This explanation however appears unlikely since one would expect to observe released virus from the cloned cells at some time; this has never been observed. A more feasible explanation may be that there is a block in either the cell or virus which is lifted by a downshift in temperature.

VI. List of Abbreviations

A	adenine
Å	angstrom (10^{-10} meter)
B	breast cells
BS-C-1	Biological standard-cercopithecus (monkey cell line)
BHK	Baby hamster kidney-21, clone 13 cells
BHKpi	Baby hamster kidney-21, clone 13 cells persistently infected with mumps virus
C	Centigrade or cytosine
CC	Cell culture
CEF	Chick embryo fibroblasts
CM-PBS	Calcium and magnesium-containing phosphate-buffered saline
CMF-PBS	Calcium and magnesium-free phosphate-buffered saline
CPE	Cytopathic effect
d	Daltons
DIP	Defective interfering particles
DNA	Deoxyribonucleic acid
EDTA	Ethylene dinitrolotetraacetic acid
EOP	Efficiency of plating (plaquing)
F	female
FA	fluorescent antibody
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FFU	Focus forming unit
G	Guanine

g	gravity or grams
g/cc	Grams per cubic centimeter
g/l	grams per liter
h	Hour
^{3}H	Tritium
HA	Hemagglutination
Had	Hemadsorption
HCl	Hydrochloric acid
HD	Hodgkin's disease
L	L cells (mouse)
Log_{10}	Logarithm base 10
LN	Lymph node
M	Male
MEM	Minimal essential medium
min	Minute
ml	Milliliter
mm	Millimeter
moi	Multiplicity of infection
mw	Molecular weight
MuVo	Wild-type mumps virus
MuVpi	Mumps virus from persistently infected BHK-21 cells
NaCl	Sodium chloride
ND	Not determined
NDV	Newcastle disease virus
NHL	Non-Hodgkin's lymphoma
Non-HD	Non-Hodgkin's disease

nm	Nanometer (millimicron, mμ, 10^{-9} meter)
PBS	Phosphate-buffered saline
PFU	Plaque forming units
PFU/ml	Plaque forming units per milliliter
RK-13	Rabbit-kidney 13 cells
RNA	Ribonucleic acid
RPM	Revolution per minute
S	Svedberg units (or spleen)
SDS	Sodium dodecyl (Lauryl) sulfate
SSC	Standard saline citrate
SSPE	Subacute sclerosing panencephalitis
Syn cells	Synovial cells
$TCID_{50}$	Tissue culture infective $dose_{50}$
TEN	Tris-HCl, EDTA, NaCl buffer
ts	Temperature-sensitive
U	Uracil (or units)
μ	Micron (10^{-6} meter)
μCi/ml	Micro Curie per milliliter
μg	Micrograms
UV	Ultraviolet
VSV	Vesicular stomatitis virus
w/v	Weight to volume
WI-38	Wistar Institute-38 cells (diploid human lung cells)
X	microscopic multiplication factor

VII. Acknowledgements

The author wishes to express sincere thanks to his major advisor, Dr. Jules V. Hallum, who contributed both scientific guidance and moral support throughout his graduate career.

I also wish to thank the members of my thesis committee, Drs. Hallum, Burger, Siegel, and Iglewski, for their scientific advice and unselfish contribution of support and time.

Additional thanks go to the members of the laboratory during my graduate studies, Drs. Robert Armen, James Evermann, and Catherine Laughlin, and to Pamela Wampler, Leslie McKitrick, and Karen Bersack for their continued assistance both scientifically and technically.

Thanks also go to N.L. Tartar for a research fellowship awarded to ALT, and to the American Society for Microbiology for a President's Fellowship awarded to the author while at the University of Oregon Health Sciences Center.

The author also expresses a very special thanks to his parents for their support and sacrifices and equally importantly for their scientific counsel.