STUDIES ON A PERSISTENT INFECTION OF MEASLES VIRUS IN HeLa CELLS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

I. Statement of the Problem

Measles virus is a member of the paramyxovirus group. The virion contains ribonucleic acid (RNA) which has a molecular weight of 6.0×10^6 daltons. The diameter of these spherical virions is 100 to 300 nm, as determined by electron microscopy. The virus is sensitive to ether and to extremes in pH. Measles virus is often referred to as a "cell associated virus". *In vivo*, measles virus latency has been related to subacute sclerosing panencephalitis (SSPE), a slow demylinating disease of the central nervous system. Measles virus has been also implicated indirectly with multiple sclerosis. Knowledge of the control of the persistence could provide the necessary *in vitro* model in understanding the pathogenesis and potential elimination of chronic human diseases where the etiology is suspected of being due to a latent virus.

In our laboratory, a persistent infection of measles virus was initiated by inoculation of a dilute virus preparation into HeLa cells. The purpose of this thesis was to understand the persistence by:

I. Characterization of the HeLa cell culture persistently infected with measles virus.

II. Characterization of the virus released from persistently infected cultures to the virus used to initiate the persistence.

II. Literature Review

A. Historical Background

The clinical disease of measles was first described by the Syrian physician, El Yehudi (48), in the year 68 B.C. Measles was thought to be a milder clinical form of smallpox until Rayes (48), a Persian, finally distinguished it 1,000 years later. Very little was added to the measles literature until the 19th century, probably because it seemed to be a less important illness when compared to such infectious diseases as the plague, smallpox, and syphilis. Being primarily a disease of children, the obvious influence on the course of world events was minimal. However, measles was responsible for the death of the heir to the French throne and resulted in the ascension of Louis XV (48).

In 1846, an outbreak of measles was reported in the Faroe Islands between Shetland and Iceland which was originated by an Icelander who developed measles after returning from Copenhagen. From this one case, an epidemic started that affected more than 6,000 people of a total population of 7,782. A Danish physician, Dr. Panum, realized that the only people spared were those who had measles as children 65 years earlier. His analysis of the epidemic made it possible to derive basic information concerning the incubation period, prodromal state of infection, clinical manifestations, along with the immunology, epidemiology, and prevention of measles (48). During the next 100 years, his observations were confirmed and extended. The next major step in the understanding of measles came in 1954 when John Enders of the

Harvard Medical School in Boston, Massachusetts isolated a filterable agent from the blood, taken on the first day of a rash from a 13 year old boy, by the name of John Edmonston. The causative agent of measles or rubeola was later characterized as measles virus (8).

B. Measles virus structure

Measles virus examined under the electron microscope is pleomorphic. The virions range in size between 100-300 nm in diameter, contain nucleocapsids of 16-18 nm in diameter and approximately 1.06 μ in length and are surrounded by a lipid membrane. Complement-fixing antibody activity has been associated with the nucleocapsids. Viral spikes extend from the virion envelope and measure 10 nm in length. These glycoprotein spikes are responsible for the hemagglutination (HA) of monkey erythrocytes. The lipid envelope as well as the hemagglutinin protein are required for infectivity and hemolysin (HL) activity. Measles virions do not elute spontaneously from red blood cells and do not contain neuraminidase molecules. The infectivity of measles virus can be completely destroyed by stirring with ether for 15 min at room temperature or after 10 min at pH 2, 3, 4, and 11. β-radiation also completely inactivates measles virus suspended in liquid medium at a range between 3-5 x 10^5 roentgen equivalent physicals/h (28). Infectivity can be maintained at temperatures below -20 C in the presence of protein for several months. However, in the absence of a protein stabilizer, only 20% of the initial infectivity is retained after two hours at 25 C.

1. Viral RNA

RNA molecules isolated from paramyxoviruses such as Newcastle disease virus (NDV) or mumps virus have a sedimentation value of 50S but this value varies among authors for measles virion RNA. Measles virus RNA, extracted by the phenol sodium dodecyl sulfate (SDS) method and centrifuged in sucrose gradients containing SDS, has been shown to co-sediment with NDV RNA, a known 50S molecule (50). Measles virus RNA has also been reported to sediment faster than NDV (14) or Simian virus-5 (SV-5) RNA (45) with a sedimentation rate of 52S. When extracted with SDS plus phenol, 10-25% of the measles virus RNA counts remained acid insoluble after hemolysis with pancreatic or T1 RNase (50). However, only 2.7% of the counts remained resistant when measles virus RNA isolated from nucleocapsids was heated and quick-cooled immediately prior to pancreatic RNase treatment. The base composition of viral RNA isolated from purified nucleocapsids by mole percent was: C(22.23 $\pm$ 0.93), A(26.45 $\pm$ 0.75), G(22.56 $\pm$ 0.75), U(28.74 $\pm$ 0.79) (49). On the basis of the nucleotide composition and RNase sensitivity, the nucleocapsid RNA of measles virus appears to be single stranded.

2. Viral proteins

Hall and Martin (10) have isolated six polypeptides from purified measles virions. The molecular weights (M.W.) determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were:

Polypeptide number	M.W.
1	75,600
2	69,000
3	60,000
4	53,000
5	51,000
6	45,700

Two (no. 2 and 4) of the six virion proteins were found to be labeled with [^{3}H]-glucosamine indicating that glycoproteins are involved in the measles virion structure. After bromelain (a proteolytic enzyme) treatment of purified virions only these two glycoproteins are removed. In addition, electron microscopy of enzyme treated virions reveals smooth, spikeless virions. This suggests that these polypeptides are associated with the surface spikes. When Tween 20 was employed to solubilize purified measles virus preparations, the two glycoproteins were found with envelope material which sedimented heterogenously between 4 and 10S. HA and HL activity were restored when the Tween 20 was removed by dialysis (11).

Bussell et al. (1) have compared the proteins of measles virus with those of canine distemper virus (CDV). Six major polypeptides were found in both measles virus and CDV preparations by SDS-PAGE (78,000, 69,000, 59,000, 51,000, 44,000, 38,000). When [^{3}H]-amino acid labeled CDV virions were co-electrophoresed with [^{14}C]-amino acid labeled measles virus, the six polypeptides had similar electrophoretic

mobilities. Only the 78,000 M.W. polypeptide from measles virions was determined to be a glycoprotein, while Hall and Martin (10) demonstrated that two measles polypeptides (no. 2 and 4) with molecular weights of 69,000 and 53,000 were labeled with [^{3}H]-glucosamine. When [^{3}H]-amino acid labeled measles virions were dissociated without mercaptoethanol (ME), a major peak of radioactivity was detected at a position which indicated a molecular weight of 185,000. Under these same non-reducing conditions, polypeptide no. 2 was not detected and a decrease in the amount of radioactivity in polypeptide no. 1 was noted. These results suggest that this large polypeptide is a virion protein which can be reduced to at least two peptide chains, one of which co-migrated with polypeptide no. 1 and the second giving rise to polypeptide no. 2. Further, Bussell's polypeptides no. 2 and no. 3 were shown to be phosphoproteins.

The characteristics given to these polypeptides by Bussell and co-workers were:

Polypeptide number	Est. M.W.	Characteristics
0	185,000	Major component minus ME
1	78,000	Glycoprotein
2	69,000	Phosphoprotein
3	58,000	Phosphoprotein nucleocapsid
4	51,000	Minor component
5	44,000	Glycoprotein in CDV
6	38,000	M protein (membrane)

Polypeptide no. 1 of measles virus has been postulated by Bussell et al. (1) to function in a similar manner as the L polypeptide of vesicular stomatitis virus (VSV) (7) which has been shown to be involved in primary viral transcription. The analogy between these proteins is based on the similar molecular weights. Polypeptide no. 6 is believed to be a non-carbohydrate containing membrane protein similar to the M protein of Sendai virus described by Scheid and Choppin (44).

3. Viral nucleocapsids

Measles virus nucleocapsids consist of approximately 2,000 copies of polypeptide no. 3 with a single measles virus genome (10). The buoyant density of isolated nucleocapsids ranges from 1.30 to 1.31 g/cc in CsCl (10,32). The sedimentation coefficient ranges from 205S to 300S (10,20,32). The viral RNA makes up approximately 5% of the nucleocapsid and has a sedimentation coefficient of approximately 52S (10). The diameter of the nucleocapsid depends on the method of preparation but averages between 16-18 nm, with the hollow inner core measured at 4.3 to 5.2 nm determined by electron microscopy (49). Measles virus nucleocapsids were estimated by Nakai et al. (29) to be 1.06 μ and 1.15 μ in length. From these measurements, measles nucleocapsids are longer than those of SV-5 (1.02 μ) and equal in length or longer than NDV nucleocapsids (1.06 μ) as reported by Compans and Choppin (5). Measles virus nucleocapsids appear rigid and tightly coiled in electron micrographs when proteolytic enzymes are used in

the isolation procedure. In the absence of proteolytic enzymes, loose, flexible nucleocapsids are seen (49). In addition, SDS-PAGE revealed that nucleocapsids harvested from infected cells in the absence of proteolytic enzymes have a single major polypeptide of 61,000 daltons. Nucleocapsid preparations harvested from infected cells with trypsin or pronase contained two polypeptides of 38,000 and 24,000 daltons.

4. Viral envelopes

The measles virus envelope consists of virus-specific proteins and host derived lipid material obtained at the time of virus budding. Nucleocapsids align themselves beneath the plasma membrane and bud outward from the cell membrane, pinching off from the cell surface to form viral particles. These pleomorphic virions contain projections or "spikes" which are glycoproteins. Electron micrographs reveal that these glycoproteins (Hall and Martin's [10] proteins no. 2 and 4) are removed from the virion surface after protease treatment. The resulting spikeless virus particles possess only minimal amounts of infectivity, HA or HL (11).

The functional role of the envelope components and glycoproteins in measles virions has been determined by the use of Tween 20, which solubilizes lipid components (11,32). All viral activities are lost in the presence of Tween 20. However, Sephadex G-200 gel chromatography of solubilized viral envelopes can separate the Tween 20 from the glycoprotein and lipid containing fractions. When the glycoprotein and lipid fractions are concentrated and allowed to re-associate, the

biological activities of HL and cell fusion are recovered.

Solubilized viral envelopes from [^{3}H]-glucosamine labeled virions, when centrifuged to equilibrium in CsCl, resulted in a single band of radioactivity and HA activity at a density of 1.26 g/cc. No cell fusion or HL activity could be detected. When these purified glycoproteins were mixed with known amounts of virus-derived lipid material or phosphatidylethanolamine, active HL and cell fusing activities were restored (11). These authors concluded that the source of the lipid component in the virus is not important since non-viral phosphatidylethanolamine restored cell-fusing and HL activities. Furthermore, Hall and Martin suggested that the lipid material may function only to maintain the proper structural alignment for the glycoproteins in the virus envelope.

Hall and Martin (12) have further separated the measles virus glycoproteins by sedimentation on linear sucrose gradients. The two glycoproteins have a sedimentation value of 6 to 7S and 9S. The larger glycoprotein (9S) was found to be responsible for the HA activity of measles virus. However, both glycoproteins and viral lipids were necessary for HL activity.

C. Measles Virus Replication

Rapp (39) has studied the growth cycle of measles virus in BSC-1 cells and found it similar to that of other paramyxoviruses. In BSC-1 cells (a continuous line of African green monkey kidney cells) infected with the virulent Edmonston strain, peak titers

($>10^5$ plaque forming units/ml [PFU/ml]) of extracellular and cell-associated virus occurred 48 h post infection. Extracellar and cell-associated virus quantities were comparable. With attenuated Schwartz strain, extracellular virus titers (10^4 PFU/ml) were less than 5% of the total yield. Nakai et al. (29) also observed a slow development of CPE similar to that obtained with attenuated measles virus strains as well as low titers of released measles virus (10^4 PFU/ml) when BSC-1 cells were inoculated with undiluted passage inoculum (multiplicity of infection, moi = 0.01-1.0).

When actinomycin D (0.1 µg/ml) was incorporated into the fluid medium, increased virus titers were observed over control cultures (27). This increase in titer was suggested to result from the suppression of interferon by the drug. This assumption was substantiated indirectly when equal viral titers were demonstrated with or without actinomycin D in Vero cells. Vero cells have been shown not to produce interferon (6). Another antibiotic, mitomycin C, has also been shown to increase virus yields and to reduce the latent period of measles virus in BSC-1 cells (40).

The cellular sites where measles virus components have been observed (and thus interpreted as representing sites of viral replication) clearly depend on the viral inoculum. By electron microscopy, Nakai et al. (29) observed that BSC-1 cells infected with measles virus which had been serially passed undiluted contained primarily intranuclear viral tubules. In addition, the lack of visible budding from these infected cells was reflected in the low virus yields

(10^4 PFU/ml). It appeared to these authors that the intranuclear tubules were trapped within the nucleus and were unable to migrate to the cytoplasmic membrane of the cell where release of the virus by budding occurred. When BSC-1 cells were infected with measles virus grown by dilute passage (moi 0.01-1.0), the tubules were found predominantly in the cytoplasm, there was extensive budding, and the titers of released virus were high (10^7 PFU/ml). These cytoplasmic tubules were hypothesized to have a greater probability of alignment beneath the cell surface and subsequently becoming infectious measles virions. Furthermore, Chiarini and Norrby (4) have reported the presence of two different sizes of nucleocapsids in measles virus infected cells. When LU-106 (human lung) cells infected with measles virus which had been passed undiluted were observed by electron microscopy, they were found to contain intranuclear nucleocapsids which were 16 to 19 nm and cytoplasmic tubules of 26 to 28 nm in diameter. Several viruses isolated from biopsied material of patients with subacute sclerosing panencephalitis (SSPE), referred to as SSPE viruses, have also been shown to produce both "smooth" (14-18 nm) and "rough" (20-35 nm) nucleocapsids (34). The significance of these two different sizes of nucleocapsids in measles virus as well as SSPE virus stocks is unknown.

Portner and Bussell (36) used an RNA inhibitor, 6-azauridine and a protein synthesis inhibitor, cyclohexamide, to study the replication of measles virus in AV-3 cells. The growth cycle of measles virus in AV-3 cells was found to be similar to that for other paramyxoviruses but different in the length of the eclipse period. Since

viral RNA and protein synthesis, necessary for virus infectivity, were first detectable between 5 and 8 h after infection and proceded mature virion production by 10 to 12 h, the authors suggest that the long eclipse phase of measles virus, when compared to other paramyxoviruses, could be due to a long maturation process and not to the somewhat slower RNA and protein synthesis of measles virus.

The finding that infected cells that have been irradiated or treated with actinomycin D or mitomycin C are still able to support measles virus replication suggests that there is no nuclear involvement with measles virus replication (23,27,40). Schluederberg and coworkers (2,3,46) could not detect RNA dependent RNA polymerase activity in nuclei isolated from infected cells while an increase in host RNA synthesis was observed. In addition, alpha-amanitin (10 µg/ml), a drug which binds to cellular RNA polymerase II, or cordycepin (50 µg/ml), an analogue of adenosine, did not affect the production of infectious virus (46) while actinomycin D (1.0 µg/ml), an inhibitor of DNA to RNA transcription, did reduce measles virus titers by 100-fold compared to non-drug treated infected Vero cells. The decreased viral titers were postulated to result from the action of actinomycin D on the viral replicative intermediate and not on nuclear functions (2). It is still unclear, however, what functions, active or passive, can be ascribed to the nucleus of a cell infected with measles virus.

D. Measles Virus Variants

In 1961, Oddo et al. (33) reported two different types of cytopathic effect (CPE) in HeLa cells from the same initial stock of

measles virus. The "strand-forming" CPE was noted after undiluted passage of virus inoculum (10° line or UP virus stock), while the "giant cell" CPE formed after diluted passage (10^{-2} line or DP virus stock). Virus stocks from strand-forming cultures re-acquired the giant cell CPE upon diluted passage. The giant cell CPE could revert to strand-forming CPE when 10^{-2} line virus stocks were passed undiluted. These types of CPE were also demonstrated in KB and primary monkey kidney cells. Infectious virus titers were always 1 to 2 $\log_{10}$ higher in 10^{-2} line stocks than 10° line virus stocks. The "giant cell" variant induced early and rapid CPE, when compared to the "strand-forming" variant. The "strand-forming" variant often initiated persistent viral infections (33). Only the UP variant was found to interfere with poliovirus replication. The ratio of plaque formation to tissue culture infective dose ($TCID_{50}$) revealed that the UP viral stocks rarely formed plaques in HeLa cells while causing cell destruction in HeLa cell roller tube cultures. The DP viral plaque assay titers, on the other hand, always correlated with CPE. If UP virus plaques were picked, virus stocks exhibited the giant cell type CPE.

The HA and HL activities of the UP and DP virus stocks were also studied. Non-concentrated tissue culture fluid from cells inoculated with UP virus demonstrated HA or HL titers from 1:32 to 1:128. No detectable HA or HL titers were found in similarly treated culture fluids taken from cells infected with DP virus.

These authors postulated that an autointerference phenomenon as noted by Hsiung (17) or Johnson and McLaren (18), or interfering

factors (particles) present in high titers, were responsible for the lack of plaque formation by UP virus. As will be shown later, Hall et al. (13) have demonstrated that slower sedimenting measles virus particles, which are both defective and interfere with normal virus replication, are present in virus stocks which have been passed undiluted.

Seligman and Rapp (47) have also observed the strand-forming or spindle cell type of CPE with measles virus passaged in human amnion or Hep-2 cells. Initially, giant cell CPE was present; however, upon subsequent passage, the spindle cell formation CPE became predominant.

In 1970, Chiarini and Norrby (4) reported on the separation of the DP and UP virus variants. Concentrated UP and DP virus fluids centrifuged to equilibrium in CsCl resulted in peaks of infectivity at 1.24 and 1.25 g/cc, which demonstrated both HA and HL activity. In addition, a second non-infectious peak of HA and HL activity was found only in the UP virus preparations at a density of 1.20 to 1.21 g/cc. When examined by electron microscopy, most of these lighter density particles appeared to be empty envelopes. However, a similar 1.20 to 1.21 g/cc peak was observed after equilibrium centrifugation of concentrated culture fluids from a persistent infection of LU-106 cells (4). However, these lighter density particles contained some nucleocapsid material.

Electron micrographs of UP virus infected cells regularly contained intranuclear nucleocapsid structures often to the extent

that paracrystalline arrangements were seen (4). The DP virus infected cells revealed mostly cytoplasmic nucleocapsid structures with little nuclear involvement. These cytoplasmic structures were described as "filamentous" coiled structures with a large diameter (27-35 nm) for measles virus nucleocapsids. These filamentous ("rough") nucleocapsids were found mostly in infected cells where intranuclear tubules were absent. Therefore, the virus passage history affects the density of the virion, the type of CPE, the relative titer of infectious virus produced and the ability of the virus to establish a carrier culture.

In addition, the virus passage history affects the synthesis of viral RNA in infected cells. Parafonovich, Hammarskjold and Norrby (35) determined the sedimentation characteristics of virus-specific RNA synthesized in Vero cells infected with either the UP or DP measles virus variants. Five days after infection, the DP variant infected cells demonstrated maximal CPE while the UP infected cultures had only minimal CPE. The [^{3}H]-uridine labeled RNA species extracted from DP infected cells during this same time period sedimented at approximately 52S, 32-34S, and 16-20S. Virus-specific RNA isolated from UP infected cultures, 7 to 9 days after infection, was minimal in quantity and could not be distinguished on the basis of sedimentation.

The RNA species isolated from cells infected with DP virus were similar in size to previously published reports. With the UP virus, this was the first time the correlation between the low level of CPE and virus production was made with viral RNA synthesis. The

authors offered the explanation that the decrease in RNA synthesis with the UP virus may be a result of defective virus replication, a pattern which was already noted with cells infected with influenza virus stocks passed according to Von Magnus (35).

Schluederberg (45) has reported that measles virus passaged in diluted form contains RNA which has a sedimentation coefficient of 52S when compared to the 50S virion RNA of SV-5. In addition to the 52S RNA, smaller virion RNA species, referred to as "subgenomic" (21S, 28S and 35S), were found in a measles virus preparation after three undiluted passages. These "subgenomic" RNA species isolated from purified measles virions were found only in UP virus stocks. Viral RNA of approximately the same sedimentation values was isolated from cytoplasmic extracts of UP virus infected cells. It was noted that these virus-specific RNA species, which had sedimentation values similar to those isolated by Parfanovich et al. (35) from DP-virus infected cells, appeared sequentially and accumulated at different rates.

Hall et al. (13) demonstrated that measles virus, passed undiluted three (UP-3) or eight (UP-8) times, contained, in addition to the normal sized measles virions, slow sedimentating particles which contained subgenomic RNA species. These particles, separated by rate zonal centrifugation on sucrose gradients, were non-infectious and interfered with production of infectious virus (defective interfering particles). These UP virus stocks demonstrated the same type of cellular destruction described earlier by Oddo et al. (33). One class of

these lighter particles contained nucleocapsids that sedimented at 130-150S and contained only 18S RNA molecules. Wild-type measles virus (DP) nucleocapsids, in comparison, sediment at 260-280S and contain only 52S RNA molecules (10).

Kiley, Gray and Payne (20) also found shorter measles virus nucleocapsids from Vero cells infected with the Edmonston strain of measles virus passed undiluted. 200S, 170S, and 110S nucleocapsids were isolated from cytoplasmic extracts of infected cells. The 200S nucleocapsids contained 50S RNA molecules of approximately 6.0×10^6 daltons. The 170S nucleocapsids were found only in small amounts and were not characterized, while the 110S nucleocapsids contained 16S RNA molecules of 0.6×10^6 daltons.

These recent findings by Kiley et al. (20) and Hall et al. (13) demonstrate that a population of defective interfering particles of measles virus have a smaller RNA molecule encased by the nucleocapsid protein. These defective interfering particles become more prominent as measles virus stocks are passed undiluted. The effects of these particles on wild-type measles virus replication and CPE were noted early by Oddo et al. (33), Rapp (49), and Norrby and co-workers (4,31).

E. Measles Virus Persistent Infections

Rustigian was the first to report on a persistent infection of measles virus (41). Since that time numerous studies concerned with measles virus persistence have appeared in the literature. This

review will attempt to describe the similarities and differences between measles virus persistent infections *in vitro*. It will be concerned primarily with the establishment and maintenance of the persistently infected cultures along with the characteristics of the virus-cell interaction.

The establishment of measles virus persistence usually begins with inoculation of a susceptible cell culture with the Edmonston strain of wild-type measles virus (30,42). However, the attenuated vaccine strains of measles virus have also been used to initiate carrier cultures (19,21). A small number of cells survive the lytic infection and begin to multiply approximately one month after infection. These cells are then cultured and passed routinely. Measles antibody is not required to initiate a persistent infection although it has been incorporated into the liquid medium of measles infected cultures possibly selecting for a highly cell-associated virus variant (9,43).

Little information has been reported on the precise nature of the viral population used to initiate persistent infections. The manner in which the virus is passaged, either undiluted or diluted before infection, has just been recognized as being important. The presence of defective interfering particles (DIP) in the virus inoculum has not been studied due to difficulties in their isolation. Neither has the possible role of temperature-sensitive (ts) mutants in establishing persistent infections been examined. These points should be evaluated when latent infections are established in the

future because of the recent reports from Hall et al. (13) on the presence of DIP in measles virus stocks and by Youngner and colleagues (37,38) on the selection of ts mutants from wild-type stocks in their persistent infections of NDV and VSV.

The cells used in measles virus persistent infections can vary from primary chicken (51) or hamster (21) embryo fibroblasts, to diploid WI-38 cells (22), to continuous lines of HeLa (26,42), Hep-2 (9), LU-106 (30) and BGM (24) cells. Why certain cells are referred to as "abortive hosts" which readily initiate carrier states while others are so "permissive" that they are destroyed by the virus is unknown. Other factors such as the temperature of incubation, the position in the cell cycle, and whether the cells are in suspension or monolayer form may influence the establishment of persistence. More information is obviously needed to define the role of the host cell in the establishment and maintenance of measles virus persistence.

The characterization of viral properties in cells persistently infected has been extensive. After the persistently infected cell cultures have stabilized, the presence of viral components can be observed by immunofluorescence (IF) or by hemadsorption (HAD). With measles virus, only monkey erythrocytes can be used as an assay for the viral hemagglutinin glycoprotein. The percentage of cells which were positive by HAD ranged between 1% in the BGM/MV cells (24) to 95% in the LU-106 persistent infection (30). By IF, 10-15% of the chronically infected chicken embryo fibroblasts (51) to 100% of the persistently infected HeLa cells (42) contained measles virus antigens.

Both nuclear and cytoplasmic fluorescence can be observed in certain virus-cell systems (9,42).

When tested, persistently infected cell cultures replicated as efficiently as uninfected control cells with a minimum of CPE visible by light microscopy. Infected as well as uninfected cell clones could be derived from parental cultures (26,42).

No significant levels of interferon or interferon-like substances were produced from HeLa or LU-106 cells persistently infected with measles virus (30,42). In contrast, by the use of a microtiter technique, Menna et al. (25) were able to detect 2 U/ml of interferon from the culture fluid of the BGM/MV cell line.

Cells persistently infected with measles virus were not resistant to superinfection with heterologous viruses, such as polio, herpes simplex, vaccinia (42), VSV or mumps virus (25). However, homologous interference could be demonstrated when measles virus was inoculated into persistently infected cell cultures (25,30,42). These results indicated that autointerference was present in the persistently infected cells which was not mediated by the action of the interferon.

Infectious center determinations performed by plating persistently infected cells on susceptible cell monolayers indicated that approximately 10% of the cells released infectious virus (21,26).

Both non-productive (24,43) and productive (26,30) persistent measles virus infections have been reported. Rustigian investigated the effect of viral antibody on a persistent infection of HeLa cells by measles virus (43). Two sublines of persistently infected HeLa

cells were grown in the presence of human measles antisera. After 39 passages, a non-productive state developed in these cells. No infectious virus could be detected from culture fluids or cell homogenates when assayed in HeLa cell cultures, although the effect of temperature was not studied. There was, however, continued synthesis of intracellular viral antigens detected by immunofluorescence. Menna et al. (25) have also described a non-productive persistent infection of an African green monkey kidney cell line, BGM cells, with mouse adapted measles virus (Edmonston strain). These cells, referred to as the BGM/MV cell line, ceased production of infectious virus 20 days after establishment. Subsequent BGM/MV cell lysates were also negative for infectious measles virus while 100% of the cells demonstrated intracytoplasmic measles virus antigens by immunofluorescence. Ultraviolet irradiation, incubation at 30 C for 36 h, or co-cultivation of BGM/MV cells with BGM cells failed to induce the production of infectious virus. Plaque assays for infectious measles virus were performed on BGM monolayers grown at 36 C.

The virus released from persistently infected cell systems has been compared in most cases to the virus used to initiate the persistence. HA and complement fixation (CF) tests performed directly on cell fluids from persistently infected cultures were negative (25,42) while cultures infected with wild-type measles virus demonstrated 80-320 HA units and 10 CF units (42). HA and CF as well as HL activity could be demonstrated when cell culture fluids from chronically infected cells were concentrated 10-fold by polyethylene glycol (30).

The virus obtained from the HeLa cell persistent infections demonstrated minimal CPE when inoculated into HeLa cells (26,42). In fact, a carrier culture could readily be established from the virus taken from Minagawa's HeLa/MV cell line (26). In all cases, the chronically carried virus titers (both extracellular and cell-associated) were between 10^1 to 10^5-fold lower than those observed for the same number of cells infected with wild-type measles virus (30,32). When the growth cycle of wild-type measles virus was compared to persistently infected virus, a significantly slower rate of virus production was obtained for the virus produced from the persistence in either HeLa or Vero cells; however again, the effect of temperature was not studied. The decrease in the growth rate of the virus from persistently infected cells was not due to thermolability since the rate of heat inactivation was similar when compared to wild-type measles virus (26).

Measles virus produced from several persistently infected cell lines has been shown to be temperature sensitive (9,15,30). When the LU 106 carrier cells were incubated at 33 C a significant increase in CPE, HA activity, and virus production was observed over carrier cultures maintained at 37 C. A latent infection of hamster embryo fibroblasts by measles virus produced 1 infectious virus per 20,000 cells when incubated at 37 C. Virus production was increased to 1 infectious virus per 200 cells when the cells were maintained at 33 C, while no infectious virus was released when cells were grown at 39 C (21). Haspel et al. (15) confirmed that the virus obtained from these latently infected hamster fibroblasts was temperature

sensitive. Gould and Linton (9) have also demonstrated the presence of a temperature sensitive virus in their measles virus persistence of Hep-2 cells.

The comparison of virion RNA from wild-type measles virus to the virus taken from persistently infected cells has not been performed. However, Winston, Rustigian and Bratt (50) extended the earlier work of Rustigian to include an analysis of the virus-specific RNA synthesized in persistently infected cells. A clone of persistently infected cells (K11) and a subclone grown in the presence of measles antisera (K11-HG-1) both contained the RNA species (50S, 30 to 35S, and 18S) found in the lytic infection of measles virus (Edmonston strain) in HeLa cells. This finding is unusual in that the subclone K11-HG-1 produces no infectious virus but still synthesizes the major 50S virion RNA species. Two other subclones, a virus producing subclone (K11-VP-1) and a non-productive subclone (K11-VF-1) were compared to the K11 clone and to a wild-type infected culture. The intracellular virus specific RNA for the productive subclone (K11-VP-1) was similar to both those of the K11 clone and cells infected with wild-type virus, while the RNA isolated from the non-productive subclone was similar to that of uninfected HeLa cells. Three times more virus-specific RNA was isolated from HeLa cells infected with wild-type measles virus than obtained from persistently infected cell cultures although the sedimentation patterns were similar. From these results the authors concluded that no additional information was obtained concerning the measles virus persistence in HeLa cells.

It is our hope that we can characterize the virus produced in the persistently infected HeLa cells and try to correlate our findings to the viruses isolated from chronic human disease, like SSPE, where a variant of measles virus is suspected of being the etiological agent.

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III. Paper 1.

Characterization of a Persistent Measles Virus Infection in HeLa Cells

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Abstract

A persistent infection with the Edmonston strain of measles virus was established in HeLa cells in the absence of measles virus antibody ($HeLa_{PI}$ cells). Between 80 and 100% of the cells possessed measles virus components as detected by hemadsorption or immunofluorescence. $HeLa_{PI}$ cells produced no interferon and were resistant to superinfection with wild-type measles virus (MV_O) but not to Newcastle disease virus and vesicular stomatitis virus. $HeLa_{PI}$ cells contained both smooth (15-18 nm) and rough (20-35 nm) nucleocapsids. Infected as well as uninfected cells could be cloned from the parental $HeLa_{PI}$ cell culture. The virus produced from the $HeLa_{PI}$ cells (MV_{PI}) varied in titer between 1.5×10^2 and 5.5×10^4 PFU/ml, had a smaller plaque size and was more heat resistant than MV_O. The eclipse and latent periods were longer for MV_{PI} compared to MV_O, however both the released and the cell-associated viral titers were approximately the same.

Introduction

Rustigian (20) and Minagawa (14) demonstrated that persistent infections of HeLa cells could be established with measles virus. The virus isolated from the persistently infected cells differed from the wild-type measles virus used to initiate the persistence by the lack of cytopathic effect (CPE) for HeLa cells and by the low viral titers produced from infected HeLa or Vero cells (14,20).

A persistent infection of HeLa cells was established with the Edmonston strain of measles virus. This report describes the establishment of the persistence, the biological properties of the virus associated with the infected HeLa cells and a comparison of the growth curve, heat inactivation and plaque size between MV_O and MV_{PI}.

Materials and Methods

Cells. BSC-1 and Vero cells, both continuous lines of African green monkey kidney cells, as well as HeLa cells, were cultured in Eagle's minimal essential medium (MEM) with 10% heat inactivated (56 C for 30 min) fetal calf serum (FCS) and sodium bicarbonate (1.0 g/l). All media contained 100 U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were incubated at 37 C unless otherwise indicated. Once monolayers were confluent, or after virus infection, the cells were maintained in the above media with 2% FCS. Cell cultures were screened for mycoplasma contamination as described by Hayflick and Stanbridge (8).

Virus. The Edmonston strain of measles virus (MV_0) was obtained from the American Type Culture Collection (Rockville, Md.). Virus stocks were prepared in Vero cells by adsorbing diluted (10^{-2}) passage material for 2 h at 33 C. When infected cells showed maximal cytopathic effect (CPE), cultures were frozen and thawed twice, centrifuged at 10,000 x g for 30 min to remove cellular debris, and stored at -70 C. The measles virus stock used to establish the persistence had been passed two times at a multiplicity of infection (moi) of less than 0.01 in BSC-1 cells before infection in HeLa cells.

Hemagglutination (HA) and complement fixation (CF) assays. Serial two-fold dilutions of viral suspensions in phosphate-buffered saline (PBS, 0.01 M phosphate, pH 7.2) were made in 0.2 ml volumes. Each tube then received an equal volume of a 1% suspension of washed Rhesus monkey erythrocytes. The results were determined after incubation at 37 C for 30-45 min. The HA titer was expressed as the reciprocal of the highest dilution which demonstrated complete hemagglutination. Measles virus HA antigen obtained from a commercial source (Flow Lab, Rockville, Md.) served as a positive control. A CF microtiter technique was kindly performed by E. de Zubizeratta as described in detail elsewhere (1,24).

Hemadsorption. Hemadsorption (HAD) assays were performed according to Rustigian (20). Suspensions of infected cells were mixed with washed Rhesus monkey erythrocytes to give a red blood cell to HeLa cell ratio of approximately 50. The cell mixtures were then incubated at 37 C for 30 min, centrifuged at 200 x g for 5 min, and resuspended. The percentage of infected cells was determined after observation of at least 200 cells in a hemocytometer by light microscopy. $HeLa_{pI}$ cells were considered positive for HAD when three or more monkey erythrocytes were observed to be attached to a cell.

Hemadsorption was also performed on cell monolayers. The medium was removed, the cells rinsed two times with PBS and a 1% suspension of Rhesus monkey erythrocytes was added. The cultures were incubated at 37 C for 30 min, rinsed thoroughly and observed. Uninfected cell monolayers served as controls.

Infectivity titrations. A plaque assay previously described for measles virus (7) was employed with modifications. Vero cells were grown in 60 mm plastic petri dishes at 37 C in an atmosphere of 5% CO_2. Confluent monolayers were rinsed with warm PBS and inoculated with 0.5 ml of virus dilution made in MEM plus 2% FCS. After adsorption at 33 C for 2 h, 7 ml of nutrient agar solution was added to give a final concentration of 10% FCS, 1% agar, and 0.23% sodium bicarbonate. The plates were incubated for 7 days in the presence of 5% CO_2 after which 3 ml of a 1:30 dilution of neutral red in PBS from a 1:300 dilution stock (Gibco, Santa Clara, Calif.) was added. The plaque forming units/ml (PFU/ml) were determined after 8-10 days.

Alternatively, tissue culture infective doses ($TCID_{50}$) assays were performed in serial ten-fold dilutions. Five roller tubes of BSC-1 or Vero cells per dilution were inoculated with 0.2 ml of the virus suspensions and observed daily for CPE. Titers were calculated 10 days after infection by the Reed and Muench method.

Cell viability determination. Cells were diluted 1:2 into a 0.5% trypan blue solution in PBS. Cells which excluded the trypan blue were considered viable.

Cell generation time determination. The cell generation time of HeLa and $HeLa_{pI}$ cells was determined at 37 C. 2-oz. glass bottles were seeded with 1 x 10^6 viable HeLa or $HeLa_{pI}$ cells. The cells were cultured in MEM plus 10% FCS. At each time interval, 2 bottles of HeLa

or HeLa$_{pI}$ cells were rinsed with PBS and trypsinized from the bottle. The cells from two bottles were combined, diluted in 0.5% trypan blue solution and counted in a hemocytometer. The viability for both HeLa and HeLa$_{pI}$ cells after trypsinization was greater than 99%.

Infectious center assay. To determine what percentage of HeLa$_{pI}$ cells were releasing infectious virus, HeLa$_{pI}$ cells were rinsed three times with PBS and removed from the flasks by scraping with a rubber policeman, pelleted by centrifugation, rinsed, re-centrifuged, and plated on Vero cell monolayers. This procedure had been used by Knight et al. (10) and Thacore and Youngner (22) in assays of persistent infections. As noted by Rustigian (20), scraping resulted in poor HeLa cell viability during routine passage, so trypsinization with an alkaline trypsin (0.05%) versene (ATV) solution was used to remove the cells for infectious center determination. HeLa and HeLa$_{pI}$ cells scraped from monolayer cultures were between 60 to 80% viable while cells that were trypsinized were greater than 99%. The procedure employed after passage 64 (P64) was according to Crowell and Syverton (5). HeLa$_{pI}$ cell monolayers were rinsed three times with PBS and trypsinized or rinsed three times with PBS and allowed to incubate with human measles convalescent antisera for 30 min at 37 C, rinsed three times again with PBS and then trypsinized from the monolayer. After trypsinization, the cells were diluted and plated on Vero cell monolayers. After 1 h at 33 C to allow attachment of HeLa$_{pI}$ cells, the plates were overlaid with nutrient agar mixture as described above

for the plaque assay. The percentage of cells that attached was not determined. Extracellular virus released after the scraping procedure varied from 150 PFU/ml for 4.5×10^5 $HeLa_{PI}$ cells to 9,300 PFU/ml for 1×10^7 $HeLa_{PI}$ cells. This number was subtracted from plaques produced by each cell suspension. The addition of the measles antisera neutralized this extracellular virus. Since the release of virus from $HeLa_{PI}$ cells during the incubation period could not be prevented, the numbers given for infectious centers were considered to be the maximum.

Thermal inactivation. Viral samples were diluted 1:10 in PBS and submerged in a water bath at 42 C or 50 C. At the indicated times, an aliquot was removed and placed on ice until all the samples could be assayed by plaque formation in Vero cells. Protein determinations were performed by the method of Lowry (11).

Growth curve of MV_O and MV_{PI} in Vero cells. Roller tube cell cultures of Vero cells were infected with MV_O or MV_{PI} at an input moi of 0.05. After virus adsorption, the cells were rinsed three times with PBS and maintenance media added. At indicated intervals, extracellular fluid was harvested and assayed for released virus. After the cells were rinsed three times with PBS, cell-associated virus was harvested after two cycles of freezing at -70 C and thawing. Infectivity assays were performed in Vero cells at 37 C with four plates per dilution.

Cell clones.

The passage of cell clones designated MJ, EE, and RZ was performed with ATV solution. Approximately 10^2-10^3 cloned cells from microtiter tissue culture plates (96 flat bottom wells, Falcon Plastics, Oxnard, Calif.) were transferred to 60 mm petri dishes which contained approximately 3 x 10^6 cells at confluency. This was designated as P1 to P2. After P2, the cell clones were subcultured from 1-60 mm petri dish to 4 or 5 new dishes.

Interferon assay. Supernatant culture fluids or clarified cell lysates from infected monolayers were assayed for interferon by the plaque reduction method using vesicular stomatitis virus (VSV) (6). Samples to be assayed were dialyzed for 48 h at 4 C against glycine buffer (pH 2.0). The fluids were then neutralized and centrifuged three times at 105,000 x g in a Spinco fixed angle 30 rotor for 1 h or continuously for 14 h. In certain experiments, fluids were harvested and treated with guinea pig anti-measles virus antisera and then centrifuged. Infectious measles virus was not detected in either preparation. Half-log dilutions of treated fluids were incubated with HeLa cells or U cells (a continuous line of human amnion cells) in plastic 60 mm petri dishes (3 ml/dish) for 18 h in a 5% CO_2 incubator at 37 C. After incubation, the cells were washed twice with PBS and challenged with approximately 50 PFU of VSV per dish. Plaques were counted after 48 h. The endpoint of the assay was taken as the reciprocal of the dilution calculated to give a 50% reduction in plaque number. Human interferon samples obtained from T. Merigan

(Stanford University) served as positive controls. The lower limit of detection by this assay was estimated to be 10 U/ml.

Immunofluorescence (IF). Coverslip cell cultures were rinsed with PBS and fixed with 95% methanol for 5 min at room temperature. The coverslips were rinsed again and covered with human subacute sclerosing panencephalitis (SSPE) serum at a 1:100 dilution (HI titer, 1:1024) kindly supplied by D.A. Fuccillo (NINDS). After 1 h at 37 C, the cells were rinsed with PBS and stained with a 1:20 dilution of sheep anti-human globulin conjugated to fluorescein isothiocyanate (Sylvana Co., Milburn, New Jersey) for 1 h at 37 C. The coverslips were then counterstained with Evans blue (0.06%) for 5 min at room temperature, rinsed thoroughly, mounted and observed with a Zeiss microscope (Reichert illuminator). Photomicrographs were taken on Kodak high speed Ektachrome film (ASA 160) with an exposure time of 2-3 min.

The specificity of this reaction was determined by a series of control experiments (12). Positive fluorescence was not detected when SSPE serum was reacted with uninfected HeLa cells, uninfected WI-38 cells, uninfected BHK-21 cells, BHK-21 cells infected with mumps virus (23), and HeLa cells infected with Newcastle disease virus (NDV). Fluorescence was not removed after adsorption of SSPE serum with uninfected HeLa cells, uninfected WI-38 cells, uninfected BHK-21 cells, or BHK-21 cells infected with mumps virus. The fluorescence was removed by adsorption with $HeLa_{PI}$ cells or after extensive absorption with a concentrated measles virus preparation grown in HeLa cells or

primary African green monkey kidney cells. Additionally, positive fluorescence was observed when SSPE serum was reacted with WI-38 cells infected with measles virus or with a carrier SSPE cell line obtained from J. Evermann (4). Fluorescence was not observed when only the conjugate was added to uninfected or persistently infected HeLa cells.

Electron microscopy. Cell monolayer cultures were rinsed twice with PBS and fixed in situ for 1 h at room temperature with 1.4% glutaraldehyde and 1.5% formaldehyde in 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 PBS and centrifuged again. The washed cells were post-fixed in 1% (w/v) osmium tetroxide in PBS for 1 h at room temperature. The cells were then washed twice in 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. (The electron microscopic studies were performed in collaboration with R. Brooks of the Department of Pathology, UOHSC).

Homologous interference challenge of $HeLa_{PI}$ cell cultures with MV_O. Monolayer cultures of HeLa and $HeLa_{PI}$ cells, P5, were grown to confluency in roller tubes. At confluency, each roller tube culture was infected with MV_O at a moi of 0.01, allowed to adsorb for 2 h at 33 C in 0.2 ml, rinsed two times with PBS after which 2 ml of maintenance media were added. After 48 h, the supernatants from duplicate

cultures were pooled and centrifuged to remove cell debris. Culture fluids were assayed by endpoint method in BSC-1 cells prior to the development of the Vero cell plaque assay.

Heterologous interference of HeLap$_I$ cultures with Newcastle disease virus and vesicular stomatitis virus. HeLa and HeLap$_I$ cell cultures were grown in 60 mm petri dishes (2.5-3.0 x 10^6 cells). At confluency the cells were infected with NDV or VSV at a moi of 0.1 or 0.01. After a 1 h adsorption at 37 C, the cells were rinsed thoroughly with PBS and maintenance media was added and incubated at 37 C in the presence of 5% CO_2 for 24 h. The supernatant fluids were collected and the cellular debris removed by low speed centrifugation. The virus samples were stored at -70 C and assayed by plaque formation in chicken embryo fibroblast monolayers (6). In a second group of challenge experiments with NDV only, HeLa and HeLap$_I$ cells grown in plastic trays (Flow Lab, Rockville, Md.) containing 24 wells of 16 mm in diameter (6.0-7.0 x 10^4 cells/well) were infected at moi's of 4, 1, 0.1, and 0.01. After 24 h at 37 C, cell cultures were frozen and thawed twice before plaque forming units per ml were determined.

Results

Establishment and initial characterization of the $HeLa_{PI}$ cell cultures. The establishment of this persistent infection was performed in the absence of measles virus antibody by inoculation of measles virus (Edmonston strain) onto HeLa cell monolayer cultures (moi 0.01). Multinucleated giant cells as well as spindle-type CPE were observed through the first 14 days post infection. By 35 days most of the cells in the culture had been destroyed. Cells which survived and grew into a monolayer were termed $HeLa_{PI}$ cells. At 37 C, $HeLa_{PI}$ cells were shown to have the same generation time of 23 h as uninfected HeLa cells determined by cell counts. $HeLa_{PI}$ cells stained with hematoxylin and eosin had the same epitheloid morphology as uninfected HeLa cells except for the occasional presence of a multinucleated giant cell. As previously described (20), the 0.25% trypsin or alkaline-trypsin-versene solution used to passage $HeLa_{PI}$ cells did not affect the percentage of cells that hemadsorbed or altered the titer of released infectious virus.

Between P3 and P7, $HeLa_{PI}$ cell suspensions demonstrated that 80-90% of the cells were HAD positive (Table 1). When HAD was measured on $HeLa_{PI}$ cell monolayer cultures, the fraction of hemadsorbing cells increased to 100%. This HAD could be blocked by treatment with guinea pig anti-measles virus antisera but not with normal guinea pig serum. Immunofluorescence of $HeLa_{PI}$ cultures between P25 and P28 revealed that virtually every cell had measles virus antigens. An average of 30% of

the HeLap$_I$ cells from P25 to P28 demonstrated cytoplasmic fluorescence exclusively while 10% stained only in the nucleus. Approximately 60% of the infected cells showed both cytoplasmic and nuclear fluorescence (Fig. 1). The reason for the different cellular locations of fluorescence is unknown at this time.

Challenge of HeLa and HeLap$_I$ cell cultures with exogenous viruses. To determine if HeLap$_I$ cell cultures were resistant to homologous viral challenge, a property of a regulated type of persistent infection (25), MV$_O$ was superinfected into HeLap$_I$ cell cultures (P5) and uninfected HeLa cell controls. After 48 h, cell culture fluids were harvested and assayed. The results are given in Table 2. Wild-type measles virus replicated efficiently in HeLa cells. However, there was no increase in the usual titer of virus released from HeLap$_I$ cells. In addition, no CPE was observed in HeLap$_I$ cell cultures while approximately 50% of the HeLa cell monolayer was destroyed. The virus obtained after MV$_O$ challenge of HeLap$_I$ cells was not further analyzed.

Persistent infections of measles virus have been reported not to inhibit the replication of exogenous viruses such as vaccinia or poliovirus (20). HeLap$_I$ cells were challenged with VSV, a rhabdovirus, and NDV, a fowl paramyxovirus. The results (Table 3) indicate that NDV and VSV replicate efficiently in HeLap$_I$ cells at a moi of 0.1 or 0.01. In the second group of experiments with NDV in which the cell cultures were frozen and thawed to release cell-associated virus, the virus

titers from $HeLa_{pI}$ cells were slightly higher than those for control HeLa cells. The absence of detectable levels of interferon in $HeLa_{pI}$ cells could explain these observations. However, these findings indicate that heterologous viral replication does take plate in $HeLa_{pI}$ cells but that release of the virus is slightly inhibited, possibly at the cytoplasmic membrane and that by freezing and thawing of the infected cells results in the release of this virus. In this regard, similar results were obtained by Menna et al. (13) using a persistent infection of measles virus/BGM cells.

Interferon production in $HeLa_{pI}$ cells. Interferon has been reported to be involved in the maintenance of certain persistent viral infections in vitro (9,22). Persistent infections established with measles virus have not been studied for the presence of interferon by standardized procedures. By the plaque reduction method employing VSV as the challenge virus, no detectable levels of interferon were found when $HeLa_{pI}$ cell fluids or cell lysates were assayed between P3 and P9. In addition, no interferon was found when culture fluids were assayed 2, 4, 9, or 14 days after subculturing. These results suggest that viral persistence in this system is not maintained by the action of interferon.

Electron micrographs of $HeLa_{pI}$ cells. $HeLa_{pI}$ cells (P21-P25) were examined for the presence of measles virus components by electron microscopy. $HeLa_{pI}$ cells revealed budding virus particles and the

presence of two different sizes of nucleocapsids observed previously in SSPE virus infected cells (16). The smooth nucleocapsids measured approximately 15 to 18 nm in diameter while the rough nucleocapsids measured approximately 20 to 35 nm in diameter. Of the HeLap$_I$ cells examined both the smooth and rough nucleocapsids were found in 20% of the cells at the same time, the smooth tubules being located only in the nucleus while the rough filaments appeared only in the cytoplasm. In the other cell preparations observed, 60% contained only rough cytoplasmic nucleocapsids, while the remaining 20% had only smooth intranuclear tubules. The smooth nucleocapsids were frequently observed in a paracrystalline alignment within the nucleus of HeLap$_I$ cells (Fig. 2).

Infectious center assay. To approximate the number of HeLap$_I$ cells that released infectious virus, serial ten-fold dilutions of HeLap$_I$ cells scraped from monolayer cultures were plated on Vero cell monolayers and overlaid with agar as described for the plaque assay. Neutral red was added after seven days and plaques counted. The results of five separate experiments with P20 and three assays with P64 HeLap$_I$ cells revealed that one in eight to one in sixteen HeLap$_I$ cells formed a plaque in Vero cells (Table 4). However, when HeLap$_I$ cells (P70 and P75) were trypsinized from cell monolayer cultures, the percentages of infectious centers increased to approximately 50%. This increase in the number of plaque forming cells could be explained in part by a decrease in cell clumps or by an increase in HeLa cell

viability due to the trypsinization procedure (19).

HeLap$_I$ cell clones. After P20, HeLap$_I$ cells were plated at limiting dilutions to establish cloned cell lines. Seven of nine cell clones established were positive for HAD. Three selected clonal lines (MJ, EE and RZ), which were greater than 95% HAD positive at P1, were analyzed for measles virus components by IF, for released virus, and for infectious centers (Table 5). After initially demonstrating greater than 95% HAD, these viral properties were not detected in clonal lines RZ and MJ after P2 and P7 respectively. All uninfected cell lines were susceptible to wild-type measles virus infection. After P6 clonal line EE resembled the parental HeLap$_I$ cell culture, when titered for infectious virus, the percentage of cells carrying measles virus antigens or infectious center determination. The disappearance of the viral antigens in clones MJ and RZ, detected by HAD or IF, could be explained by non-replicating viral components which were diluted with continued cell replication. Other interpretations are also possible.

Properties of the virus (MVp$_I$) isolated from HeLap$_I$ cell cultures. HeLap$_I$ cell fluids and lysates were assayed routinely for the presence of infectious virus by plaque assay in Vero cells. The viral titers ranged between 1.5×10^2 and 5.5×10^4 PFU/ml. The ratio of released or cell-associated virus per HeLap$_I$ cell for a 24 h period ranged between 1 to 5 virions per 10 cells for released virus and 1 to 7

virions for cell-associated viruses (Table 6). Because of the low viral titers no correlation could be established between measles virus production and rapidly proliferating cell cultures as has been shown for the hamster embryo fibroblast-measles virus system (7).

Measles virus HA or CF assays performed on $HeLa_{PI}$ cell fluids were below detectable levels (20). MV_{PI} does not cause extensive CPE in HeLa cells when monitored by light microscopy. In fact, MV_{PI} readily establishes a persistent infection in HeLa cells at a moi of 0.2 with less than 10% of the cells showing CPE. Similar results have been reported by Minagawa (14). However, cell destruction is observed in Vero cells with MV_{PI}. This CPE can be blocked by prior treatment of MV_{PI} with guinea pig anti-MV_O antisera.

MV_O and MV_{PI} differ in plaque size on Vero cell monolayers incubated at 37 C. Wild-type measles virus plaques, 8 days after infection, measure 2.0 mm or larger in diameter, while MV_{PI} plaques measure 1.0 mm or less in diameter. This difference in plaque size can clearly be seen in Figure 3.

MV_O and MV_{PI} virus stocks were compared for the ability to resist thermal inactivation at 42 and 50 C. The results of three separate experiments at 42 C are given in Table 7. After 60 min at 42 C, MV_{PI} retained 66, 60 and 37% of its initial infectivity, compared to 2, 7, and 0.8% of MV_O survival. At 50 C for 15 min, the titer of MV_O decreased more than 100-fold while the MV_{PI} titer dropped less than 10-fold (Table 8). Clearly MV_{PI} is more resistant to heat inactivation at 42 or 50 C than MV_O. This difference is not due to viral

aggregates since in the second experiment $HeLa_{PI}$ cell fluid was taken directly from cell cultures and subjected to 42 C. Sonication was also employed in the third experiments to both MV_0 and MV_{PI} to minimize viral clumps. No significant differences in protein concentration could be detected between MV_0 and MV_{PI} (third experiment) by Lowry determination. This finding is not surprising since a heat resistant variant of VSV has been reported by Petric and Preuc (18). A group of temperature sensitive mutants of measles virus have also been shown to be more heat stable than wild-type measles virus (B. Fields, personal communication).

Replication of MV_0 and MVp_I in Vero cell monolayer cultures. The growth curve of MV_0 and MVp_I in Vero cell cultures is shown in Figure 4. At 37 C, cell-associated virus was initially detected in MV_0 infected cultures 18 h after infection and 24 h after infection for MVp_I. MV_0 was first released into the medium between 18 and 24 h post infection while released MVp_I was observed between 24 and 36 h. Upon release, both MV_0 and MVp_I titers increased at similar rates; however the maximum released MV_0 titer (96 h) was approximately 100-fold greater than for MV_{PI}. Both MV_0 and MV_{PI} cell-associated titers were approximately equal. After 48 h of infection, approximately 50% of the MV_0 infected cell monolayer demonstrated CPE while only 10-20% CPE was observed in MV_{PI} infected cell cultures. Vero cells infected with MV_{PI} released approximately 10^3 PFU/ml 7 days after infection, while MV_0 viral titers dropped after 5 days.

Discussion

The HeLa$_{pI}$ cell line was established by inoculation of wild-type measles virus into HeLa cell monolayers. Measles virus CPE was observed 3 days after infection and lasted for 35 days. The cells which repopulated the bottle were morphologically similar and had the same generation time as uninfected HeLa cells. These HeLa$_{pI}$ cells have been maintained in this laboratory since February 1973.

After P15, HeLa$_{pI}$ cell monolayers revealed that essentially every cell contained the viral hemagglutinating glycoprotein as detected by HAD of Rhesus monkey erythrocytes. Measles virus antigens were located in either the cytoplasm, nucleus or both of HeLa$_{pI}$ cells by IF.

In a persistent infection of L cells with NDV, 10-30 units of interferon were continually produced (22). This interferon blocked the replication of VSV when superinfected. In contrast, no interferon was detected in the HeLa$_{pI}$ cell system. HeLa$_{pI}$ cells were capable of being superinfected and permitted NDV and VSV to replicate efficiently. However, when challenged with wild-type measles virus, the viral titer did not increase above background levels. The mechanism of this homologous interference is not known but may involve blockage of receptor sites on cells by viral glycoproteins as suggested by Palma and Huang (17).

Electron microscopic studies revealed the presence of two distinct sizes of nucleocapsids. The "smooth" filaments (15 to 18 nm) were observed only in the nucleus of HeLa$_{pI}$ cells. The larger "rough"

tubules (20-35 nm) were located exclusively in the cytoplasm. These larger nucleocapsids could be observed beneath the cytoplasmic membrane of infected cells and in membranous particles adjacent to the cell surface. HeLa cells infected with measles virus have been shown to contain intranuclear tubules 96 h to 120 h after infection (15). However, the formation of extremely large nuclear inclusions comprised of smooth nucleocapsids appears to be characteristic of SSPE viruses, but not measles virus (16). If this observation can be confirmed and extended to include other SSPE virus isolates, then the large quantities of nucleocapsids found in the nuclei of $HeLa_{PI}$ cells might indicate that a virus subpopulation similar to SSPE was selected from the wild-type measles virus during the persistence.

From 6 to 13% of the $HeLa_{PI}$ cells release infectious virus when scraped from the cell monolayer. However, when brief treatment with trypsin was employed, the fraction of $HeLa_{PI}$ cells which formed a plaque increased to approximately 50%. The reason for these differences when trypsinization was used could be explained by an increase in cell viability or by release of infectious virus during the incubation period caused by the enzymatic activity on the membrane of infected cells. Considering this variability, these results were interpreted to mean that not all of the $HeLa_{PI}$ cells were releasing infectious virus.

Rustigian (20) has reported that a persistently infected clonal cell line derived from parental cultures decreased in the number of infected cells from 77% HAD positive to less than 0.1% after 29

passages. After 19 subcultures another cloned cell culture increased from 10% HAD positive to 61%. Similar results were obtained with the $HeLa_{PI}$ cell system. MJ and RZ cloned cell cultures decreased in the percentage of infected cells while cell clone EE, after P6, had identical viral properties to the parental cultures. These findings suggest that the persistence of infection in cloned cell lines is variable and that defective virus or cellular regulation could alter the synthesis of measles virus components.

MV_{PI} was released from $HeLa_{PI}$ cells in low titers (1 to 5 PFU/10 cells) while approximately the same quantity of virus was cell-associated (1 to 7 PFU/10 cells). Wild-type measles virus infection of Hep-2 cells has been reported to produce between 8 and 15 infectious virions per cell (3).

MV_{PI} causes less CPE when inoculated into HeLa cells than MV_O and could readily establish a persistent infection. When MV_{PI} from $HeLa_{PI}$ cell cultures was assayed in Vero cell monolayers, it was observed that after 8 days the plaques were all 1 mm or less in diameter. This was in marked contrast to the plaque size (2 mm or larger in diameter) of wild-type MV_O.

Heat inactivation was performed to determine if any virion structural differences could be detected between MV_O and MV_{PI}. Thermolability was determined by the loss of infectivity during the incubation of virus samples at 42 or 50 C. The results of three separate experiments at 42 C and one at 50 C clearly show that MV_{PI} is more resistant to thermal inactivation than MV_O.

The growth pattern of MV_O and MV_{PI} in Vero cells revealed that MV_{PI} replicated slower than MV_O and produced lower viral yields. In contrast to the chronically infected measles virus hamster fibroblast system (7) where a block of infectious virus production occurred at a late stage of maturation, both MV_{PI} released and cell-associated viral titers were comparable suggesting that release was not inhibited at the cell membrane.

These results indicate that additional differences exist between MV_O and MV_{PI} than those described by Rustigian (20) and Minagawa (14). It appears that these differences in the MV_{PI} population result from the $HeLa_{PI}$ cell persistence which was initiated with wild-type MV_O. The mechanism of selection of MV_{PI} by the persistent infection remains unknown but appears to select for a virus which could be similar to the viruses isolated from SSPE patients. The possible role of DNA intermediates (2) or temperature sensitive mutants of measles virus (7) in this regard is currently under investigation.

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Table 1

The percentage of $HeLa_{P_I}$ cells demonstrating measles virus components as detected by hemadsorption (HAD) or immunofluorescence (IF).

$HeLa_{P_I}$ cells passage no.	% positive cells by HAD	% positive cells by IF
3[a]	88	N.D.[b]
4	83	"
5	85	"
6	80	"
7	91	"
10	86	"
11	92	"
12	80	"
13	88	"
14	88	"
15[c]	100	"
20	100	"
25	100	100
26	100	100
27	100	100
28	100	100
30	100	100
40	100	100
50	100	100

[a] HAD performed on cell suspensions between passages 3-14.

[b] N.D. = not determined.

[c] HAD performed on monolayer cultures from passages 15-50.

Table 2

Challenge of HeLa and $HeLa_{pI}$ cells with wild-type measles virus

Virus	Cells	Titer of virus yield[a]
MV_o	HeLa	$10^{3.5}$
MV_o	$HeLa_{pI}$	$10^{1.5}$
None	$HeLa_{pI}$	$10^{1.5}$

[a] $TCID_{50}$/0.2 ml assayed as described in Materials and Methods.

Table 3

Challenge of HeLa and $HeLa_{PI}$ cells with Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV).

1st Group

Virus	Moi	Cells	Titer of virus yield[a]
NDV	0.1	HeLa	$10^{6.1}$
NDV	0.1	$HeLa_{PI}$	$10^{5.3}$
VSV	0.1	HeLa	$10^{6.9}$
VSV	0.1	$HeLa_{PI}$	$10^{6.3}$
NDV	0.01	HeLa	$10^{5.4}$
NDV	0.01	$HeLa_{PI}$	$10^{4.4}$
VSV	0.01	HeLa	$10^{5.8}$
VSV	0.01	$HeLa_{PI}$	$10^{5.3}$

2nd Group

NDV	4	HeLa	$10^{5.8}$
NDV	4	$HeLa_{PI}$	$10^{5.9}$
NDV	1	HeLa	$10^{5.7}$
NDV	1	$HeLa_{PI}$	$10^{5.8}$
NDV	0.1	HeLa	$10^{5.4}$
NDV	0.1	$HeLa_{PI}$	$10^{5.8}$
NDV	0.01	HeLa	$10^{5.8}$
NDV	0.01	$HeLa_{PI}$	$10^{6.1}$

[a] Plaque forming units per ml in chicken embryo fibroblasts.

Table 4

Infectious center determinations of $HeLa_{PI}$ cells on Vero cell monolayers[a]

$HeLa_{PI}$ cell passage no.	No. of plaques /100 $HeLa_{PI}$ cells[b]	% infectious centers
P20	7/100	7
	13/100	13
	13/100	13
	13/100	13
	10/100	10
P64	6/100	6
	6/100	6
	8/100	8
P70	53/100	53
P75	50/100	50

[a] Details of procedure are described in Materials and Methods.

[b] Average number of plaques rounded to nearest whole number.

Table 5

Properties of clonal cell lines from HeLap$_I$ cell cultures.

Cell clone	Passage no.	Property HAD[a]	IF[b]	IC[c]	PFU/ml[d]
EE	P1	95	N.D.[e]	N.D.	N.D.
	P3	25	20%	N.D.	10^3
	P6	95	95%	1 in 8	10^4
MJ	P1	95	N.D.	N.D.	N.D.
	P5	0	2%	N.D.	0
	P7	0	0	<1 in 5.3×10^3	N.D.
	P8	N.D.	0	N.D.	0
RZ	P1	95	N.D.	N.D.	N.D.
	P2	0	0	N.D.	0
	P3	0	0	<1 in 1.5×10^4	0

[a] HAD = the percentage of cells which demonstrated hemadsorption of Rhesus monkey erythrocytes.

[b] IF = immunofluorescence. The presence of measles virus antigens in cell clones as determined by immunofluorescence.

[c] IC = infectious center determination. The average number of plaques formed in Vero cells per number of cells plated.

[d] Titer of infectious virus as determined by plaque assay.

[e] N.D. = not determined.

Table 6

The ratio of released and cell-associated MV_{PI} per 10 $HeLap_I$ cells for a 24 h period[a].

$HeLap_I$ cell passage no.	Released virus PFU/10 cells	Cell associated PFU/10 cells	Total PFU/10 cells
P20	1.0	N.D.[b]	N.D.
P64[c]	1.7	2.0	3.7
P64	4.2	3.0	7.2
P64	4.7	7.4	12.1
P65	2.2	1.2	3.4
P66	5.4	N.D.	N.D.

[a] Assayed in Vero cells at 37 C.

[b] N.D. = not determined.

[c] Three separate determinations on $HeLap_I$ cell (P64).

Third experiment

Time at 42 C	MV_0 titer	Log decrease	% survivors
0 min	5.0×10^4	0	100
20 min	1.14×10^4	0.64	23
40 min	5.4×10^3	0.97	11
60 min	3.8×10^3	1.12	0.8

Time at 42 C	MV_{PI} (P63) titer	Log decrease	% survivors
0 min	4.6×10^3	0	100
20 min	4.5×10^3	0.01	98
40 min	2.4×10^3	0.28	52
60 min	1.7×10^3	0.43	37

[a] PFU/ml determined by plaque assay in Vero cells.

Table 8

Comparison of the thermolability of MV_o and MV_{PI} at 50 C.

Time at 50 C	MV_o titer[a]	Log decrease	% survivors
0 min	9.6×10^4	0	100
15 min	3.9×10^2	2.39	0.4

Time at 50 C	MV_{PI} (P21) titer	Log decrease	% survivors
0 min	2.0×10^5	0	100
15 min	2.6×10^4	0.89	13

[a] PFU/ml determined by plaque assay in Vero cells.

Figure 1.

Immunofluorescent photomicrograph of measles virus specific antigens in the cytoplasm and nucleus of HeLa cells persistently infected with measles virus (X320).

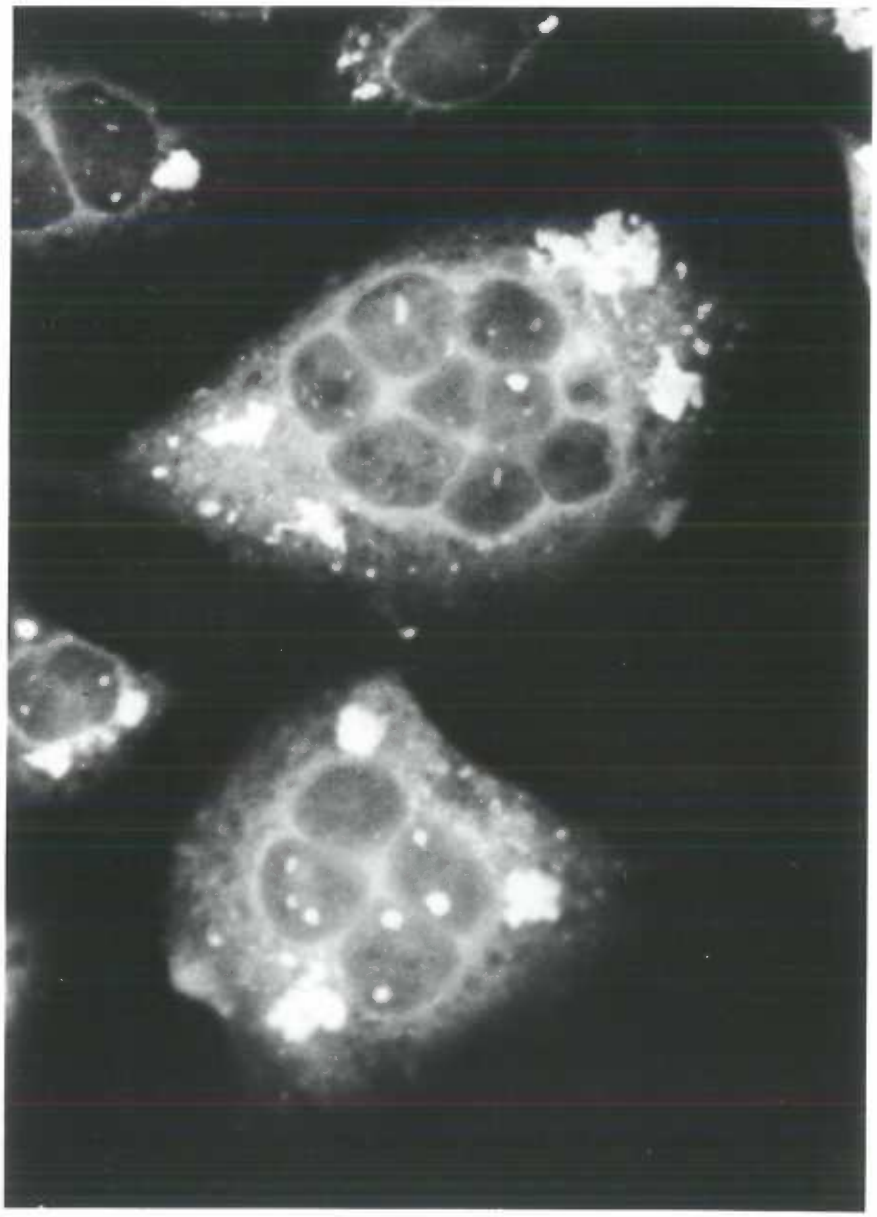


Figure 2.

An electron micrograph of a $HeLa_{PI}$ cell containing rough nucleocapsids (R) in the cytoplasm and the inclusion type of smooth tubules (S) in the nucleus. Note the intranuclear paracrystalline array (X23,400).

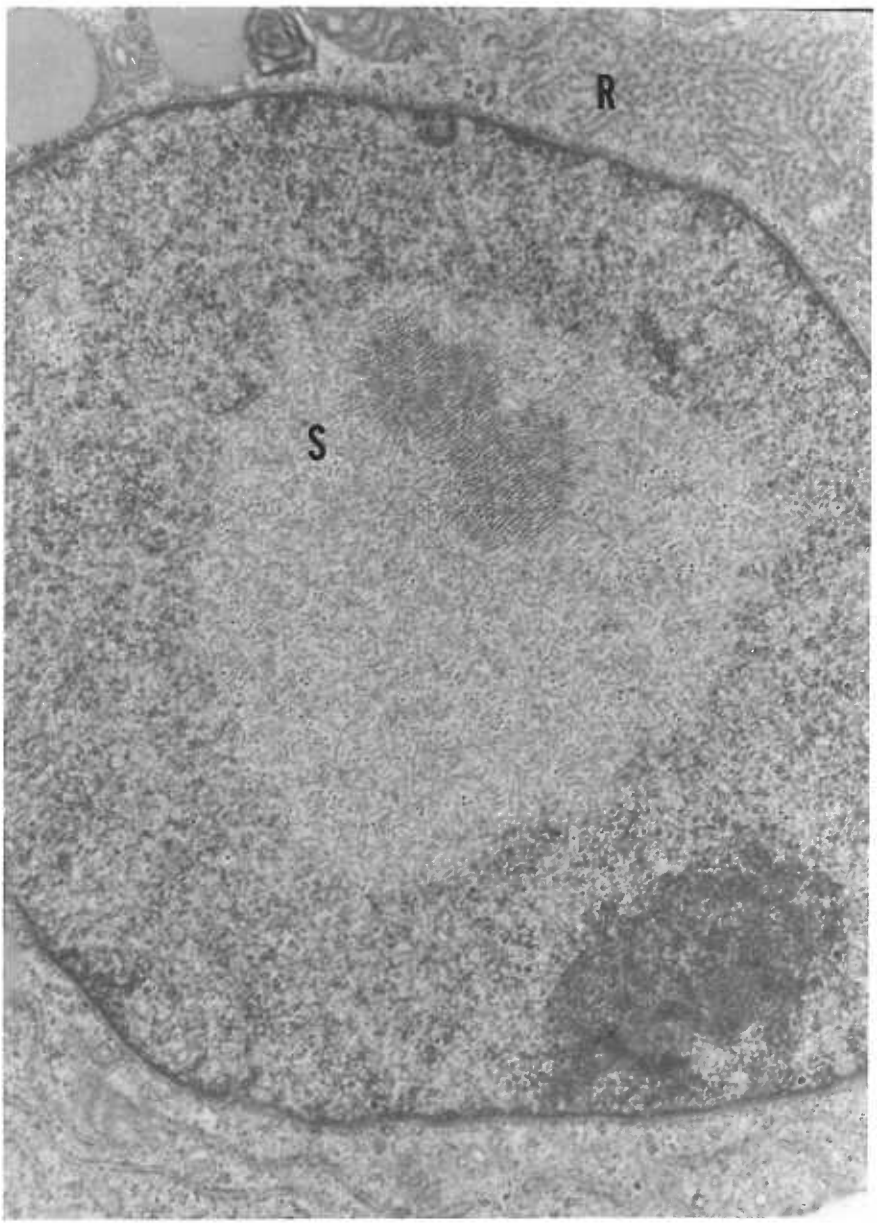

R
S

Figure 3.

Plaque size of MV_0 and MV_{PI} in Vero cells. Vero cell monolayers in 60 mm petri dishes were stained with neutral red eight days after infection. A = control plate, B = MV_0 infected plate, C = MV_{PI} infected plate.

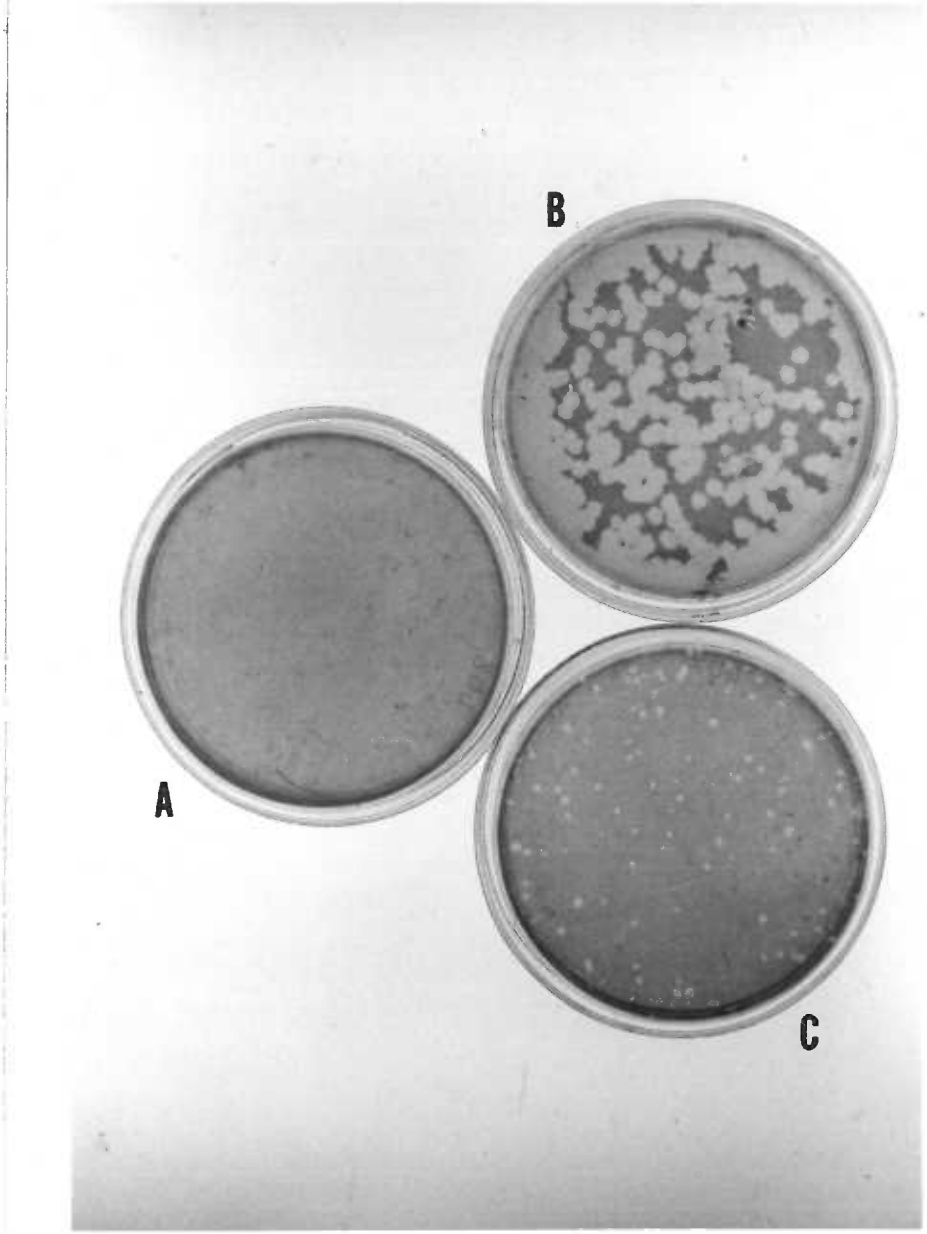
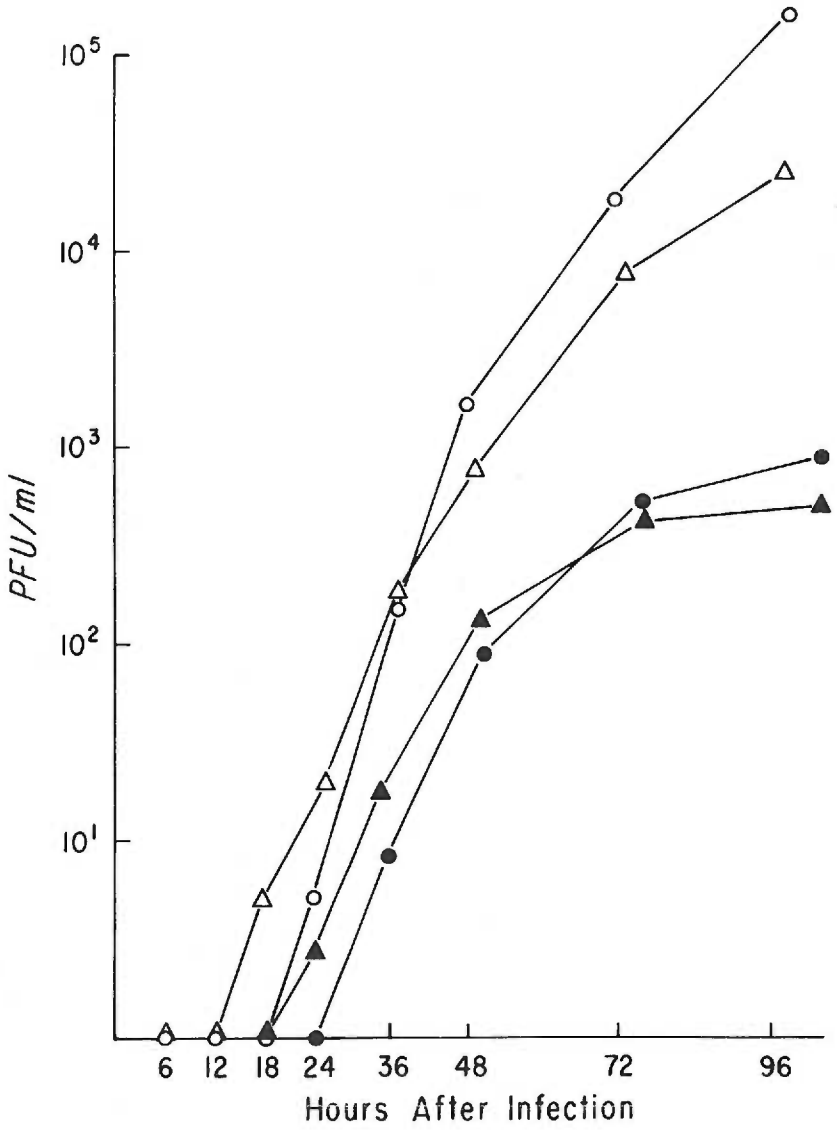

B
A
C

Figure 4.

The growth curve of MV_O and MV_{PI} in Vero cell cultures. Details of the experiment are described in Materials and Methods section. Symbols: MV_O, (○) extracellular, (△) cell-associated; MV_{PI}, (●) extracellular, (▲) cell-associated.


10^5
10^4
10^3
10^2
10^1
PFU/ml
6
12
18
24
36
48
72
96
Hours After Infection

IV. Paper 2

Temperature-Sensitive Mutants of Measles Virus Isolated from Persistently Infected HeLa Cells

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Abstract

The virus isolated from HeLa cells persistently infected with the Edmonston strain of measles virus (MV_{pI}) has been reported from this laboratory to differ from the wild-type measles virus (MV_o) in reduced virulence for HeLa cells, lower virus production from Vero cells, and increased heat stability at 42 C. Additionally, MV_{pI} has now been shown to be temperature sensitive. The temperature sensitivity of MV_{pI} was determined by the efficiency of plaquing at 33 C and 39 C in Vero cell monolayers. After two passages in Vero cells at 33 C, 93% of the plaque-purified virus stocks maintained the temperature sensitive characteristic. There was a 50-fold increase in virus production as well as a slight increase in the percentage of cells forming infectious centers in $HeLa_{pI}$ cells incubated at 33 C compared to $HeLa_{pI}$ cells grown at 37 C. MV_{pI} readily established a persistent infection in HeLa cells which also released temperature sensitive virus.

Introduction

Temperature-sensitive (ts) mutants have been isolated from numerous cell cultures and animals persistently infected with paramyxoviruses (18). Youngner and colleagues (16-18,26) have shown that ts mutants of Newcastle disease virus are involved in the persistence of L, BHK-21, and MDCK cells. With the attenuated Schwartz strain of measles virus, Haspel et al. (8) demonstrated that the viral maturation in latently infected hamster embryo fibroblasts was temperature sensitive. Anti-measles antibody has also been utilized to establish a persistent infection of measles virus in Hep-2 cells where temperature sensitive measles virus is released (6). This paper reports that a persistent infection of HeLa cells, established without virus specific antibody with the Edmonston strain of measles virus, selects for a virus population which is temperature sensitive.

Materials and Methods

Cells. Vero cells, a continuous line of African green monkey kidney cells, and HeLa cells were cultures in Eagle's minimal essential medium (MEM) with 10% heat inactivated (56 C for 30 min) fetal calf serum (FCS) and sodium bicarbonate (1.0 g/l). All media contained 100 U/ml penicillin and 100 µg/ml streptomycin. Cell cultures were incubated at 37 C unless otherwise indicated. Once monolayers were confluent or after virus infection, the cells were maintained in the above media with 2% FCS. Cell cultures were screened for mycoplasma contamination as described by Hayflick and Stanbirdge (9).

Virus. The Edmonston strain of measles virus (MV_0) was obtained from the American Type Culture Collection (Rockville, Md.). Virus stocks were prepared in Vero cells by adsorbing diluted (10^{-2}) passage material for 2 h at 33 C. When infected cells showed maximal cytopathic effect (CPE), cultures were frozen and thawed twice, centrifuged at 10,000 x g for 30 min to remove cellular debris, and stored at -70 C. The characteristics of MV_{PI} have been described previously (1). A known temperature sensitive mutant (tsT) was kindly provided by F. Rapp (Hershey, Pa.) (7). Viral stocks of tsT were prepared in Vero cells at a multiplicity of infection (moi) of 0.01 at 33 C and harvested as described above.

Hemadsorption. Hemadsorption (HAD) was performed on cell monolayers. The medium was removed, the cells rinsed two times with PBS and a 1% suspension of Rhesus monkey erythrocytes was added. The cultures were incubated at 37 C for 30 min, rinsed thoroughly and observed. Uninfected monolayers served as controls.

Infectivity assays. A plaque assay previously described for measles virus was employed with modifications (8). Vero cells were grown in 60 mm plastic petri dishes at 37 C in an atmosphere of 5% CO_2. Confluent monolayers were rinsed with warm phosphate buffered saline (PBS, 0.01 M phosphate pH 7.2) and inoculated with 0.5 ml of virus dilution made in MEM plus 2% FCS. After adsorption at 33 C for 2 h, 7 ml of nutrient agar solution was added to give a final concentration of 10% FCS, 1% agar, and 0.23% sodium bicarbonate. The plates were incubated for 7 days in the presence of 5% CO_2 after which 3 ml of a 1:30 dilution of neutral red in PBS from a 1:300 stock (Gibco, Santa Clara, Calif.) was added. The plaque-forming units/ml (PFU/ml) were determined after 8-10 days.

Alternatively, tissue culture infective dose ($TCID_{50}$) assays were performed in serial ten-fold dilutions. Five roller tubes of BSC-1 or Vero cells per dilution were inoculated with 0.2 ml of the virus suspensions and observed daily for CPE. Titers were calculated 10 days after infection by the Reed and Muench method.

Infectious center assay. To determine what percentage of $HeLa_{PI}$ cells were releasing infectious virus, infectious center determinations were performed by scraping $HeLa_{PI}$ cell monolayer cultures with a rubber policeman and plating on Vero cells as previously described (1). After passage 64 (P64), trypsinization with an alkaline trypsin (0.05%) versene solution was used to remove the cells for infectious center assays according to the procedure of Crowell and Syverton (4).

Immunofluorescence (IF). Coverslip cell cultures were rinsed with PBS and fixed with 95% methanol for 5 min at room temperature. The coverslips were rinsed again and covered with human subacute sclerosing panencephalitis (SSPE) serum at a 1:100 dilution (hemagglutination inhibition titer, 1:1024) kindly supplied by D.A. Fuccillo (NINDS). After 1 h at 37 C, the cells were rinsed with PBS and stained with a 1:20 dilution of sheep anti-human globulin conjugated to fluorescein isothiocyanate (Sylvana Co., Milburn, New Jersey) for 1 h at 37 C. The coverslips were then counterstained with Evans blue (0.06%) for 5 min at room temperature, rinsed thoroughly, mounted and observed with a Zeiss microscope (Reichert illuminator). Photomicrographs were taken on Kodak high speed Ektachrome film (ASA 160) with an exposure time of 2-3 min.

The specificity of this reaction was determined by a series of control experiments (13). Positive fluorescence was not detected when SSPE serum was reacted with uninfected HeLa cells, uninfected WI-38 cells, uninfected BHK-21 cells, or BHK-21 cells infected with

mumps virus (25). The fluorescence was removed by adsorption with $HeLa_{pI}$ cells or after extensive adsorption with a concentrated measles virus preparation grown in HeLa cells or in primary African green monkey kidney cells. Additionally, positive fluorescence was observed when SSPE serum was reacted with WI-38 cells infected with measles virus or with a carrier SSPE cell line described by Burnstein et al. (3) and obtained from J. Evermann. Fluorescence was not observed when only the conjugate was added to uninfected or persistently infected HeLa cells.

Isolation of plaque-purified MV_{pI} viral stocks. The procedure used for plaque purification of MV_{pI} is similar to those described for the isolation of chemically induced ts mutants of measles virus (2,7). The agar above well separated plaques at terminal dilutions was removed with a Pasteur pipette and suspended in 1 ml of maintenance media. After brief sonication, 0.2 ml of this material was inoculated into duplicate Vero cell monolayers in 16 mm wells in plastic trays (Flow Lab, Rockville, Md.). Identical cell cultures were incubated at 33 C and 39 C and observed for CPE. MV_o and tsT were included in each isolation series as controls.

Results

Temperature sensitivity of MV_{PI}. Persistent infections of measles virus in LU 106 cells (15), hamster embryo fibroblasts (8) and Hep-2 cells (6) have been shown to produce ts mutants. Preliminary experiments with MV_O and MV_{PI} indicated that the MV_{PI} characterized previously was also temperature sensitive. Therefore, cell culture fluids taken from $HeLa_{PI}$ cells at P11, P22, and P27, and from $HeLa_{PI}$ cell clone EE (1), as well as wild-type MV_O from Vero cells, were assayed in Vero cell monolayers at 37 C and 39 C. After adsorption at 33 C for 2 h, duplicate samples were incubated at 37 and 39 C and monitored daily for CPE. The results are given in Table 1. MV_O replicated to a titer of 10^4 $TCID_{50}$/0.2 ml at both 37 and 39 C while all MV_{PI} titers from three separate subcultures of $HeLa_{PI}$ cells were at least 100-fold lower at the elevated temperature. MV_{PI} from cell clone EE showed about a 10-fold decrease. The reduction of MV_{PI} titer at 39 C indicated that a large proportion of the MV_{PI} population was temperature sensitive.

The temperature sensitivity of MV_{PI} from $HeLa_{PI}$ cells was further analyzed by determining the efficiency of plaquing at 33 and 39 C of duplicate titrations in Vero cell monolayers. This procedure has been described previously for the isolation of ts mutants of measles virus (2,7). The efficiency of plaquing (EOP) was calculated by dividing the titer at 39 C by the titer obtained at 33 C. With MV_O grown in HeLa or Vero cells, the EOP was 1.0, while for tsT, it was

3.6×10^{-5}. MV_{PI} released from $HeLa_{PI}$ cells (P63 and P66) essentially did not plaque at the non-permissive temperature, while greater than 10^4 PFU/ml were detected at 33 C. The EOP of the two MV_{PI} stocks was 2.7 and 4.4×10^{-4} (Table 2). The EOP results for MV_{PI} are consistent with those obtained for the ts virus released from the S-2 phase of the measles virus latent infection of hamster embryo fibroblasts (8).

Since temperature sensitivity of MV_{PI} was demonstrated from the total population, plaque purification was performed to determine the stability of individual MV_{PI} stocks. To date 68 out of 73 (93%) MV_{PI} plaque-purified viral stocks have produced CPE only at 33 C and not at 39 C. In addition, tsT which has been reported not to produce CPE in BSC-1 or human conjunctiva cells at 39 C (7), did not cause CPE in Vero cells at the non-permissive temperature in our laboratory. The 5 viral stocks which demonstrated CPE at both 33 and 39 C suggested the possibility that wild-type MV_o revertants were present. These findings suggest that the majority of MV_{PI} plaque-purified stocks after being plaqued and passed one time in Vero cells remain temperature sensitive. The reversion frequency of stable MV_{PI} mutants, determined by the ratio of the efficiency of plaquing, will be reported in a separate communication.

Influence of temperature shift on $HeLa_{PI}$ cell system. $HeLa_{PI}$ cells (P69) were shifted from 37 C to 33 C. Replicate cell cultures were maintained at 37 C for comparison. After 50 days (6 cell passages), the cultures shifted to 33 C were morphologically indistinguishable by light microscopy from cultures incubated at 37 C. No interferon

could be detected in culture fluids from $HeLa_{PI}$ cells cultured at 33 C or 37 C (1).

The release of MV_{PI} was monitored after $HeLa_{PI}$ cells were shifted from 37 to 33 C. After 12 h, a 2.5-fold increase in viral titer was observed in Vero cells assayed at 33 C. However, after the $HeLa_{PI}$ cells had been passed twice at 33 C (14 days), nearly a 50-fold increase in MV_{PI} titer was observed after a 12 h period (Table 3). This increase in MV_{PI} titer from $HeLa_{PI}$ cells incubated at 33 C approximates the increase in titer reported by Norrby (15) from LU 106 measles virus carrier cultures incubated at 33 C for 15 days.

Infectious center determinations on $HeLa_{PI}$ cells assayed at 33 C on Vero cells demonstrated a slight increase in the number of infectious centers from cells grown at 33 C (Table 4).

The ability of MV_{PI} to establish a persistent infection. To compare the virulence of MV_{PI} for HeLa cells with that of MV_o, triplicate roller tube cultures (1×10^5 cells) were infected with 0.2 ml of MV_{PI} (P20) at a moi of 0.2. After adsorption, the cell cultures were rinsed, fed with maintenance media and incubated at 37 C. After 10 days, less than 25% of the HeLa cell monolayers showed CPE. Minimal CPE and cell rounding continued in the MV_{PI} infected cell culture for the next 20 days at which time cells began to repopulate the roller tubes. In contrast, MV_o infected HeLa cells demonstrated extensive CPE 7 days after infection until the monolayers were destroyed. After 45 days, surviving cell clones were observed in

the MV_o infected cultures. The MV_{PI} infected cultures were assayed for released virus and for the presence of measles virus components by HAD and IF (Table 5). The new carrier HeLa cells ($HeLa_{PI}$ II cells) were 100% infected as determined by HAD or IF. The levels of released virus were comparable to titers obtained from $HeLa_{PI}$ cells (1). The EOP (39 C/33 C) of released virus from passage 4 cells was 5.5×10^{-4}, indicating that this virus was also temperature sensitive. These results show that MV_{PI} inoculated into HeLa cells causes minimal CPE and establishes a persistent infection directly. In addition, the virus released from $HeLa_{PI}$ II cells appears to be temperature sensitive. Further characterization of this cell line is being carried out.

The lack of host-induced modification in $HeLa_{PI}$ cell system.

Host-controlled variations or host-induced modifications have been described for Sendai virus (11,21) and for NDV (5,23). 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 observed variation can be attributed to a host-induced modification. In the case of the HeLa/MV cell cultures described by Minagawa, after being passed one time in Vero cells, the carried virus lost the ability to establish persistence directly and regained the plaque size of MV_o. From these results, Minagawa (14) suggested the possibility of a host-controlled variation for the mechanism of maintenance of the HeLa/MV cultures.

To determine if the lack of virulence for HeLa cells, smaller plaque size, and temperature sensitivity of MV_{PI} was a result of a HeLa cell induced modification, MV_{PI} was passed in Vero cells. Serial passages of MV_{PI} were initiated by plating 10^4 plaque forming $HeLa_{PI}$ cells onto Vero cell monolayers in 60 mm petri dishes (2-3 x 10^6 cells) at 33 and 37 C. After 96 h, approximately 75% of the cell monolayer was destroyed. The cells which remained were scraped into the media and frozen and thawed two times and cellular debris removed by centrifugation. Subsequently, a 1:10 dilution of this material was passed and harvested as described above. After being passed three times plaque assays were performed at 33 C and 39 C. The plaque size, the EOP and the virulence for HeLa cells were determined for each passage. The results of the EOP (39 C/33 C) are given in Table 6. MV_{PI} passed in Vero cells at 33 C three times did not form plaques at 39 C indicating that the ts property of MV_{PI} was a stable mutation. However, MV_{PI} passed at 37 C maintained its ts character for only two passages as determined by the EOP. After being passed at 37 C for the third time, plaques were observed in Vero cell monolayers incubated at 39 C resulting in a higher EOP. The plaque size at 33 C for all passage material was 1 mm or less (data not shown) while the plaques seen at 39 C from passage 3 (37 C) material were 2 mm. Only this latter virus preparation caused syncytia type CPE on HeLa cells at 37 C. These results indicate that the altered characteristics of MV_{PI} are not the result of a HeLa cell induced modification. Furthermore, when plaque-purified MV_0, grown in Vero cells, was passed twice in

HeLa cells at a moi of 0.001, the EOP (1.0 and 1.1), the virulence for HeLa cells (positive), and the plaque size ($\geq$2 mm) continued to be consistent with that of the wild-type MV_0.

Discussion

Several mechanisms have been suggested to explain the establishment and maintenance of persistent infections by measles virus. Rustigian (20) concluded that cellular factors controlled viral growth through a regulatory mechanism in his measles virus-HeLa cell system. Similarly, a host-induced modification was suggested by Minagawa (14). Norrby (15) found no evidence for the presence of interferon in the measles virus carrier LU 106 cells while an interference between virulent and temperature sensitive virus was suggested as the mechanism for a latent infection of hamster embryo fibroblasts with the Schwartz strain of measles virus (8).

In the $HeLa_{PI}$ cell system, interferon was excluded as a possible mechanism for the $HeLa_{PI}$ cell persistence because no detectable levels of interferon were found. The susceptibility of $HeLa_{PI}$ cells to challenge with VSV was additional evidence that interferon, if present, was in very small quantities (1).

The stability of three MV_{PI} properties upon passage in Vero cells as well as the lack of modification on HeLa cell grown wild-type MV_o indicated that a host-induced modification was not prominent in this system. The virus which was not temperature sensitive after being passed three times at 37 C probably represented wild-type MV_o revertants rather than virus which had lost its HeLa cell modification.

By the efficiency of plaquing at 39 C on Vero cells, MV_{PI} was clearly found to be temperature sensitive. When the incubation

temperature of the $HeLa_{PI}$ cells was lowered from 37 C to 33 C an increase in both the amount of virus produced and the number of cells producing virus was observed. The lack of extensive CPE after temperature shift to 33 C is in contrast to the measles virus LU 106 carrier cultures (15) where a gradual increase in CPE was observed until cells detached completely from the bottle, 30 days after incubation at 33 C.

MV_{PI} was isolated from a persistent infection which had been initiated by the Edmonston strain of measles virus. The mechanism of selection of MV_{PI} by the persistence in the absence of measles antibody is not known. However, the persistent infection appears to select for a virus which is less virulent, temperature sensitive and has a smaller plaque size. The presence of ts mutants in persistent infections of measles virus (6,8) as well as with NDV (17,18), VSV (10; Youngner, J.S., personal communication), and Sindbis virus (22) suggests that the occurrence is not a random process.

Recently, Youngner and his colleagues (personal communication) have reported evidence which might explain how temperature sensitive mutants of VSV could be involved in the maintenance of a persistent infection of L cells. These ts mutants were determined to interfere with wild-type viral replication at both the permissive and non-permissive temperature. At the same time, an increase in the ts mutant titers was also observed. Since the ts mutants can inhibit wild-type virus replication, revertants would not become the predominant virus in the persistence. Thus, a ts virus population

selected during persistence could maintain both its mutant character and the persistent infection.

The mechanism of maintenance presented above provides a workable hypothesis by which persistence with measles virus can be studied. The ability of MV_{PI} to initiate a persistent infection in HeLa cells as well as to be isolated from persistently infected cells suggests the possibility that ts mutants of measles virus may be involved in the establishment and maintenance of persistence. However, the presence of defective interfering particles of measles virus or DNA intermediates and their role in the $HeLa_{PI}$ cell system cannot be excluded.

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Table 1

The replication of MV_{PI} from $HeLa_{PI}$ cells in Vero cells at 37 C and 39 C

Virus	$TCID_{50}$/0.2 ml at	
	37 C	39 C
MV_o	10^4	10^4
MV_{PI} (P11)	10^3	$<10^1$
MV_{PI} (P22)	10^3	$<10^1$
MV_{PI} (P27)	10^3	10^1
MV_{PI} EE	10^2	$<10^1$

Table 2

The efficiency of plaquing (EOP) of measles virus stocks at 33 C and 39 C in Vero cell monolayers.

	PFU/ml at		EOP
Viral stock	33 C	39 C	39 C/33 C
MV_0	1.2×10^5	1.3×10^5	1.0
tsT	2.8×10^5	< 10	3.6×10^{-5}
MV_{PI} (P63)[a]	2.3×10^4	< 10	4.4×10^{-4}
MV_{PI} (P66)	3.7×10^4	< 10	2.7×10^{-4}

[a] $HeLa_{PI}$ cells (8×10^6) passage 63 and 66 grown at 37 C. Cell fluid was harvested after 24 h.

Table 3

The release of infectious virus from $HeLa_{PI}$ cell cultures maintained at 33 C and 37 C.[a]

Time after shift	Incubation temperature 33 C
4 h	1.7×10^{4}[b] (3.4×10^{4})[c]
8 h	2.4×10^{4} (3.6×10^{4})
12 h	9.0×10^{4} (4.0×10^{4})
336 h[d] (14 days)	1.4×10^{6} (3.0×10^{4})

[a] $HeLa_{PI}$ celts (8×10^{6}) passage P69 grown at 37 C.

[b] PFU/ml assayed in Vero cells at 33 C.

[c] Numbers in parentheses represent infectious virus titers obtained from $HeLa_{PI}$ cell cultures maintained at 37 C.

[d] $HeLa_{PI}$ cells (8×10^{6}) between P69 - P71. Cell fluid harvested after 12 h period.

Table 4

Infectious center determinations of $HeLa_{PI}$ cells on Vero cell monolayers at 33 C.[a]

Incubation temperature of $HeLa_{PI}$ cells	% infectious centers
Passage 70[b]	
33 C	60
37 C	45
Passage 71[c]	
33 C	40
37 C	18

[a] Details of procedure are given in the Materials and Methods.

[b] $HeLa_{PI}$ cells removed by scraping.

[c] $HeLa_{PI}$ cells removed by trypsinization.

Table 5

Characterization of $HeLa_{PI}$ II cells

Cell passage no.	HAD[a]	IF[b]	PFU/ml[c]
1	100	100	2.0×10^3
2	100	N.D.[d]	9.0×10^3
3	100	100	N.D.
4	N.D.	100	7.4×10^3
			1.6×10^{4e}
			$<10^f$

[a] HAD - The percentage of cells which demonstrated hemadsorption of Rhesus monkey erythrocytes.

[b] IF - Immunofluorescence. The presence of measles virus antigens in cells as determined by immunofluorescence.

[c] PFU/ml - Plaque forming units/ml in Vero cells at 37 C.

[d] N.D. - Not determined.

[e] Plaque forming units/ml in Vero cells at 33 C.

[f] Plaque forming units/ml in Vero cells at 39 C.

Table 6

The effect of serial passage on MV_{PI} in Vero cells

MV_{PI} passage no.	Incubation temperature of Vero cells	
	33 C	37 C
P1	7.6×10^{-2}[a]	2.2×10^{-3}
P2	9.0×10^{-2}	6.2×10^{-4}
P3	9.6×10^{-4}	7.2×10^{-1}

[a] Efficiency of plaquing (EOP 39 C/33 C), the PFU/ml determined at 39 C divided by PFU/ml at 33 C.

V. Appendices

APPENDIX A

The comparison of MV_o and MV_{PI} virulence in HeLa cells detected by cytopathic effect.

The purpose of this experiment was (1) to compare the virulence of MV_{PI} and wild-type MV_o for HeLa cells and (2) to determine if MV_{PI} was capable of establishing a persistent infection in HeLa cells.

Roller tube cultures of HeLa cells (1×10^5 cells) in triplicate were inoculated with three different viral preparations. Two different MV_o virus stocks passed at low moi's, one grown in HeLa cells, the other grown in Vero cells, were used; MV_{PI} from $HeLa_{PI}$ cells (P20) served as the third viral stock. Confluent monolayers were rinsed with phosphate buffered saline and 0.2 ml of a viral dilution was added to give an input moi of 0.2. After 2 h at 33 C, the cells were rinsed and 2 ml of maintenance media was added before incubating at 37 C. CPE was measured in the cell cultures by observation under the light microscope. When 25% or less of the monolayer showed viral CPE, the results were recorded as 1+. When between 25-50% of the monolayer demonstrated CPE, 2+ was the notation, while 3+ designated 50-75% of the monolayer was involved in CPE. 4+ meant that between 75-100% of the cells showed CPE.

Two days after infection, 1+ CPE was observed in both MV_o infected cell cultures. CPE was extensive (4+) in MV_o infected HeLa cells 7 days after infection while at the same time, only 1+ CPE was noted in MV_{PI} infected monolayers. Minimal cell rounding and CPE continued in the MV_{PI} infected cell cultures for the next 10 days, after which time the infected cells began to grow and repopulate the roller tubes. In contrast, MV_o infected cultures could be seen to have surviving cell clones 45 days after infection. The MV_{PI} infected

cells were assayed for released virus and for the percentage of measles virus infected cells by hemadsorption and immunofluorescence between passage 1 and 3. The results given in Table 1 show that 100% of the cells, designated $HeLa_{PI}$ II cells, are infected as detected by hemadsorption or immunofluorescence and that between 10^3 to 10^4 PFU/ml infectious virus are released.

Conclusions

MV_{PI} inoculated into HeLa cell cultures readily established a persistent infection. MV_{PI} was less virulent for HeLa cells than MV_o as determined by CPE. MV_{PI} infected monolayers remained virtually intact while nearly complete destruction of the MV_o infected cultures was observed.

Table 1

Characterization of $HeLa_{PI}$ II cells

Cell passage no.	HAD[a]	IF[b]	PFU/ml[c]
1	100	100	2.0×10^3
2	100	N.D.[d]	9.0×10^3
3	100	100	N.D.

[a] HAD - The percentage of cells which demonstrated hemadsorption of Rhesus monkey erythrocytes.

[b] IF - Immunofluorescence. The presence of measles virus antigens in cells as determined by immunofluorescence.

[c] PFU/ml - Plaque forming units/ml in Vero cells at 37 C.

[d] N.D. - Not determined.

APPENDIX B

The generation time of HeLa and $HeLa_{PI}$ cells at 37 C.

The cell generation time of HeLa and $HeLa_{PI}$ cells was determined at 37 C. 2-oz. prescription bottles were seeded with 1×10^6 viable HeLa or $HeLa_{PI}$ cells. The cells were cultured in MEM plus 10% FCS. At each time interval, 2 bottles of HeLa or $HeLa_{PI}$ cells were rinsed with PBS and trypsinized from the bottle. The cells from two bottles were combined, diluted in 0.5% trypan blue solution and counted in a hemocytometer.

The results are given in Table 1. The formula used to calculate the cell generation time was:

$$n = \frac{\log B - \log A}{\log 2}$$

where B = final number of cells, A = initial number of cells, log 2 = 0.301 and n = the number of generations. The generation time, g, was then calculated from the equation:

$$g = \frac{t_2 - t_1}{n}$$

where t_2 = the final time, t_1 = the initial time.

By taking the HeLa and $HeLa_{PI}$ cell counts from day 3 and day 5 (logarithmic growth phase), the cell generation time was determined to be 22.8 h for HeLa cells and 23.3 h for $HeLa_{PI}$ cells.

The plating efficiency of HeLa and $HeLa_{PI}$ cells was not significantly different. This difference would be expected to be minimal since the cell generation time was determined in the logarithmic growth phase for each individual cell type.

Table 1

The cell generation time of HeLa and $HeLa_{PI}$ cells at 37 C.

Day	Cell number[a] HeLa	$HeLa_{PI}$
1	4.0×10^5	3.3×10^5
2	6.2×10^5	5.0×10^5
3	1.6×10^6	1.0×10^6
4	3.6×10^6	1.6×10^6
5	7.3×10^6	4.2×10^6
6	8.0×10^6	9.2×10^5

[a] Total number of cells per 2-oz glass bottle as determined by cell counts in a hemocytometer.

APPENDIX C

The effect of measles virus antiserum on $HeLa_{PI}$ cells.

$HeLa_{PI}$ cell cultures were grown in 25 cm^2 plastic flasks with MEM plus 2% FCS. Human measles convalescent serum obtained commercially (Flow Lab, Rockville, Md.) was heat inactivated (56 C for 30 min) and added to the culture media. The dilution of antiserum used was sufficient to neutralize the released infectious virus (1 CF unit/ml/24 h). The $HeLa_{PI}$ cell morphology was not altered by the addition of antiserum.

When the antibody medium was removed after 58 days, only 52% of the $HeLa_{PI}$ cells revealed positive fluorescence, compared to >95% in the untreated control $HeLa_{PI}$ cell cultures. After 109 days, the antibody medium was removed and the cells were transferred to coverslips for immunofluorescent examination. Neither viral antigens by immunofluorescence nor released virus could be detected. After two additional passages without antibody, the cells were again negative for measles virus specific fluorescence and no released virus could be detected. The antibody "cured" cultures were susceptible to wild-type measles virus infection.

The conclusions from this experiment are limited since normal human serum was not included as a control even though anti-measles antibodies can be detected in a majority of the normal human sera by immunofluorescence (1).

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APPENDIX D

Superinfection of HeLa and $HeLa_{PI}$ cell cultures with wild-type MV_o.

Homologous interference has previously been attempted with passage 5 $HeLa_{PI}$ cells. However, no conclusions could be made concerning the nature of the virus released from $HeLa_{PI}$ cells after challenge with MV_o. To try and determine if MV_o replication was inhibited by $HeLa_{PI}$ cells, HeLa and $HeLa_{PI}$ cell cultures (passage 40) were grown in Linbro trays (24 well, 16 mm), the cultures were inoculated with MV_o at moi's of 4, 1, 0.1, and 0.01. After 24 h, the cultures were frozen and thawed two times and the culture fluids centrifuged at 1,500 x g for 20 min to remove cellular debris. The virus samples were assayed in Vero cells by plaque formation. MV_o was monitored for by the presence of plaques of 2 mm or greater in diameter on Vero cell monolayers at 37 C. The plaque size of MV_{PI} was 1 mm or less in diameter under the same conditions.

The results are given in Table 1. MV_o was concluded to replicate efficiently in HeLa and $HeLa_{PI}$ cells since viral titers approximated each other and because large plaques (2 mm or greater) were observed on terminal dilution plates. The consistently lower viral titer obtained from $HeLa_{PI}$ cells might be the result of interference between the replication of MV_o and MV_{PI} or by the presence of defective interfering particles present inside the $HeLa_{PI}$ cells. This is in contrast to the previous homologous challenge experiment where there was no increase above the usual titer of virus produced from $HeLa_{PI}$ cells. The reason for this apparent loss of homologous interference upon passage of $HeLa_{PI}$ cells is unknown. A possible explanation is that wild-type MV_o gene products, responsible for large plaques, could be

complementing or rescuing the MV_{pI} from the $HeLa_{pI}$ cells even though wild-type MV_o replication is significantly inhibited. A similar rescue of a ts mutant of VSV by wild-type VSV has been described by Youngner and Quagliana (1).

References

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Table 1

Challenge of HeLa and $HeLa_{PI}$ cells with MV_o.

Moi	Cells	Titer of virus yield[a]
4	HeLa	$10^{5.7}$
4	$HeLa_{PI}$	$10^{5.3}$
1	HeLa	$10^{5.2}$
1	$HeLa_{PI}$	$10^{4.5}$
0.1	HeLa	$10^{6.2}$
0.1	$HeLa_{PI}$	$10^{6.0}$
0.01	HeLa	$10^{6.1}$
0.01	$HeLa_{PI}$	$10^{5.7}$
-	$HeLa_{PI}$	$10^{4.9}$

[a] PFU/ml in Vero cell monolayers at 37 C.

APPENDIX E

Establishment and preliminary characterization of a Vero cell line persistently infected with measles virus.

The establishment of a persistent infection of Vero cells by measles virus was attempted. Vero cells (2×10^7) were inoculated with a measles virus preparation (moi 2×10^{-4}) that had been passed undiluted 10 times and diluted (1:10 dilution) 8 times in HeLa cells after being received from the American Type Culture Collection. After 8 days, extensive cytopathic effect was observed which continued for the next 14 days until destruction of the monolayer was virtually complete. By 130 days after infection, a sufficient number of surviving cells had re-grown and were subsequently subcultured (passage 2). By immunofluorescence at passage 2, 100% of the cells ($Vero_{PI}$ cells) contained measles virus antigens. Table 1 presents the results of the percentage of infected cells as detected by immunofluorescence. Infectivity assays of $Vero_{PI}$ cells at passage 2 and 10 using Vero cell monolayers incubated at 37 C did not reveal any infectious virus when undiluted inoculum was plated.

Electron micrographs taken of $Vero_{PI}$ cells at passage 3 revealed the presence of nucleocapsids of the smooth variety (14-18 nm in diameter) only. These smooth tubules were observed in the cytoplasm, in the nucleus or in both areas simultaneously. The percent distribution of nucleocapsids found in the cytoplasm or in the nucleus was not determined.

At passage 12, the extracellular fluid from $Vero_{PI}$ cells incubated at 37 C was assayed for infectious virus on Vero cell monolayers at 33 C and 39 C. Infectious virus was detected at 33 C (10^3 PFU/ml), but no virus was detected at 39 C (<10 PFU/ml). The plaque size of

measles virus released from the $Vero_{PI}$ cells assayed at 33 C was 1 mm or less in diameter. Viral titers were found to increase after $Vero_{PI}$ cells were incubated at 33 C. After two passages at 33 C, $Vero_{PI}$ cells produced between 7×10^{3} to 8×10^{4} PFU/ml on Vero cell monolayers incubated at 33 C while essentially no plaques (<10 PFU/ml) were found at 39 C. The efficiency of plating (39 C/33 C) for the virus from $Vero_{PI}$ cells ranged from 1.4×10^{-3} to 1.3×10^{-4}, clearly indicating that a temperature sensitive viral population had been selected in the persistence.

Table 1

The percent of $Vero_{PI}$ cells with measles virus antigens as detected by immunofluorescence.

Cell passage no.	% positive
2	100
5	100
6	40
7	70
8	50
10	75
11	50
13	100

APPENDIX F

Assay for infectious DNA in persistently infected HeLa cells.

Zhdanov and Parfanovich (6) have reported that measles virus DNA transcripts are integrated into the cell genome of a chronically infected chicken embryo fibroblast. In addition, Simpson and Iinuma (5) have recovered infectious DNA from Hep-2 cells latently infected with respiratory syncytial virus.

To determine if measles virus RNA copies had been transcribed into DNA and had integrated into the HeLa cell DNA, transfection studies were performed with purified HeLa and $HeLa_{PI}$ cell DNA.

HeLa and $HeLa_{PI}$ cells were grown and scraped from monolayer cultures of (2.8×10^8 HeLa cells and 1.5×10^8 $HeLa_{PI}$ cells). The cells were resuspended in 1X SSC (standard sodium citrate) and 0.5% SDS along with 250 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. The nucleic acid was precipitated with 2 volumes of cold absolute ethanol and the DNA was collected on a glass stirring rod and redissolved in 0.1X SSC overnight with the aid of a small magnetic stir bar. Pancreatic RNase (boiled for 10 min) was then added to give a final concentration of 50 g/ml and then allowed to incubate at 37 C for 1 h. The mixture was then extracted 3 times with a chloroform isoamyl mixture (24:1), spooled and redissolved in a 1 ml of 0.1X SSC. The DNA went into solution only after being stirred with a magnetic stir bar. The 260/280 ratios were determined on an aliquot of the HeLa and $HeLa_{PI}$ cell DNA. The ratios of each were 1.76 and 1.77 respectively. These 260/280 ratios were similar to those used by Temin and colleagues (3,4)

who isolated infectious reticuloendotheliosis virus DNA from infected cells. The total amount of DNA was determined to be approximately 900 μg for each sample by optical density (A_{260}) determination with 1.0 O.D. unit = 50 μg double-stranded DNA.

HeLa and Vero cell monolayers were transfected by the method of Boyd and Butel (2) and Al-Moslik and Dules (1). Briefly, monolayer cell cultures were grown in Linbro 24 well plastic dishes (16 mm) to confluency (2×10^5 cells/well). The media were 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; 0.20 ml) for 10 min. The cells were then rinsed 3 times with CMF-PBS. The DNA was then added in concentration of 5 and 50 μg/well (0.25 ml) in duplicate and allowed to incubate at room temperature for 15 min. The cell cultures were then rinsed with CMF-PBS and 2 ml of maintenance media was added to each well and incubated at 37 C in 5% CO_2 atmosphere. HeLa and $HeLa_{PI}$ cell DNA (120 μg) incubated (37 C for 1 h) with 100 μg/ml (total volume 0.6 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 antigen by hemadsorption and immunofluorescence and for infectious virus for over 3 weeks and 3 cell subculturings. Neither viral antigens nor infectious virus was detected or recovered after each subculturing. These results, although negative, did not exclude the possibility that infectious DNA is present in $HeLa_{PI}$ cells.

References

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APPENDIX G

The analysis of MV_o and MV_{PI} by equilibrium centrifugation in sucrose gradients.

Plaque-purified MV_{PI} from Vero cells (MV_{PI} clone 18) was compared to MV_{PI} taken directly from $HeLa_{PI}$ cells by equilibrium centrifugation. (MV_{PI} clone 18 was shown later to be a wild-type revertant by efficiency of plaquing, plaque size, and virulence for HeLa cells.) This experiment was performed to determine if density differences existed between a virus stock derived from a single plaque compared to the virus released from the $HeLa_{PI}$ cells.

Two confluent roller bottles of Vero cells were infected with MV_{PI} clone 18 at a moi of <1. After adsorption for 2 h at 33 C, 200 ml of maintenance media was added which contained 25 μCi/ml of ^{3}H uridine. After approximately 80% of the monolayer demonstrated CPE, the cell fluids were harvested and the cellular debris removed by centrifugation at 10,000 x g for 30 min. The supernatant fluid was then centrifuged in a Spinco 30 rotor at 23,000 RPM for 3 h. The resulting pellet was resuspended and layered onto a 15-65% sucrose-D_2O gradient and centrifuged for 4 h at 39,000 RPM in a SW 41 rotor. $HeLa_{PI}$ cell cultures (passage 27) were radioactively labeled and purified as described above. The centrifuge tubes were fractionated into 0.4 ml aliquots into sterile agglutination tubes. A 10 or 25 lambda aliquot from each fraction was taken and precipitated with 1 ml of ice cold 10% TCA (trichloroacetic acid). 200 μg of bovine serum albumin was added as carrier. The tubes were then set in ice for at least 30 min at 4 C before filtration. Each sample was filtered under vacuum with two rinses of cold 10% TCA and then 70% ethanol. The filters were manufactured by either Millipore (HA) or Whatman (GF/C). The filters

were dried and placed in scintillation vials along with 10 ml of aqueous toluene based counting solution (4 g Omnifluor [NEN] in 1 liter toluene) in a Beckman LS beta counter. The linearity of the 15-65% sucrose-D_2O gradients formed from a commercial gradient maker was checked by taking 0.4 ml fractions and determining the density from the refractive index or from the percent solids in solution with an Abbe refractometer. The densities of gradient fractions were determined from the weight difference of a full minus an empty 10 lambda pipet. No significant amount of quench was determined by B/A channel ratio. The gradient peaks were unaltered by longer centrifugation (12 h).

The gradient profile of MV_{PI} clone 18 after centrifugation is given in Figure 1. A major peak of radioactivity was found at a density of 1.24 g/cc with a slight shoulder at 1.20 g/cc and one at 1.26 g/cc. MV_{PI} purified in a similar manner directly from $HeLa_{PI}$ cells demonstrated three peaks of radioactivity (Fig. 2). The MV_{PI} population was concluded to be a heterogenous viral population with major peaks at 1.20 g/cc (fraction 18), 1.24 g/cc (fraction 20), and 1.26 g/cc (fraction 22). A minor peak was observed at a density of approximately 1.30 g/cc (fraction 24).

A viral stock (MV_o) was inoculated into HeLa cells at a 10^{-1} dilution. This viral preparation was passed in an identical manner to the virus inoculum used to initiate the $HeLa_{PI}$ cell persistence. After 4 days 1+ CPE was observed. The maintenance media were replaced with medium which contained 25 µCi/ml of ^3H uridine. These media

were replaced daily until the cell monolayer demonstrated 4+ CPE. The cell fluids were clarified by low speed centrifugation and the supernatant fluid was concentrated by ultrafiltration at 4 C for 3 days (2). The concentrated sample was then centrifuged in a sucrose gradient as described above (Fig. 3). Two peaks of radioactivity were detected: a lighter peak at 1.21 g/cc (fraction 14) and one peak at 1.24 g/cc (fraction 18). This finding demonstrates that supernatant fluid from infected cultures can be concentrated without pelleting the virus and indicates that the 1.20 g/cc and 1.24 g/cc peaks are not artifacts resulting from the procedures of pelleting. Also, this graph suggests that the 1.21 g/cc peak could be involved in establishing a persistent infection and that the 1.21 g/cc peak could also be involved in the maintenance of the persistence since it can be isolated directly from $HeLa_{PI}$ cells.

After 3 to 8 undiluted passages of measles virus, Hall et al. (1) have shown that defective interfering particles of these viruses increase in number with each passage. To determine if undiluted passage caused an increase in the 1.20-1.21 g/cc peak, MV_{PI} virus clone 18 originally passed in diluted form (3 times, 10^{-2} dilution) was inoculated into Vero cells in 32 oz bottle (2×10^7 cells) at a moi of approximately 0.1 without dilution. This passage history was referred to as 1X undilute. After 12 h and maximum CPE, the culture fluid was removed and the cellular debris removed by low speed centrifugation. 5 ml of this material was then adsorbed directly into another 32 oz bottle containing Vero cells. This procedure was performed to build

up viral stocks passed undiluted 1X, 2X, and 3X. The resulting viral stocks (5 ml) were then adsorbed to Vero cell monolayers (3-32 oz bottles) for 2 h at 37 C, rinsed with PBS and maintenance media was added, which contained 25 μCi/ml of ^{3}H uridine. After 12 h all bottles demonstrated 4+ CPE. The fluids were harvested and cellular debris removed with low speed centrifugation (10,000 x g for 30 min). The fluids were then pelleted at 23,000 RPM in Spinco 30 rotor for 3 h. The pellets were resuspended in TEN (0.01 M Tris pH 7.4, 0.1 M NaCl, 0.001 M EDTA) buffer and layered on 15-65% sucrose-D_2O gradient and centrifuged at 39,000 RPM in SW 41 rotor for 4 h. The results of these gradients are given in Fig. 4, 5, and 6. All three major radioactive peaks were observed at a density of 1.24 g/cc (fraction 18). It was concluded that after 3 undiluted passages of MV_{PI} clone 18 that no 1.21 g/cc peak was observed, and if defective interfering particles were present, they had a density of 1.24 g/cc.

When paramyxoviruses are pelleted viral aggregates are formed. To dissociate these aggregates brief sonication or Dounce homogenization is routinely used. To answer the question: Does sonication result in the formation of 1.21 g/cc peak? MV_{PI} clone 18 passed 4X undiluted was adsorbed (5 ml/32 oz) onto 3-32 oz of Vero cells for 2 h at 37 C, rinsed with PBS and fed with 50 ml of maintenance media with 25 μCi/ml of ^{3}H uridine. After 12 h and maximum CPE, the fluids were harvested and cellular debris removed and then subjected to 23,000 RPM in Spinco 30 rotor for 3 h at 4 C. The pellets were resuspended in TEN and combined. The sample was then split in half. One half was

sonicated briefly (1 sec) on a Bronson Biosonic IV model no. BIO IV (low setting, min. dial). The other sample was not sonicated. The individual samples were then layered on a 15-65% sucrose gradient and centrifuged to equilibrium. Figure 7 shows the gradient profile of the non-sonicated viral fraction. A slight 1.21 g/cc shoulder is observed, indicating that the 1.21 g/cc peak formation is not caused by sonication. Figure 8 represents the gradient profile of sonicated sample with a broad 1.21 g/cc shoulder.

To analyze some of the biological properties of the virus in the 1.21 g/cc density peak, 3,000 ml of $HeLa_{PI}$ cell fluid was collected from passage 29, 30 and 31. The cellular debris was removed by low speed centrifugation (10,000 x g for 30 min) and then pelleted in Spinco 30 rotor at 23,000 RPM for 3 h. The pellet was resuspended in TEN buffer and then layered on 15-65% sucrose-D_2O gradient and centrifuged for 12 h at 23,000 RPM in an SW 27 rotor at 4 C. One milliliter fractions were collected and the absorbance at 254 nm (A_{254}) was determined. 0.2 ml aliquots from each fraction was diluted with 1.8 ml of TEN buffer and read in Beckman spectrophotometer. The gradient profile is given in Figure 9. Hemagglutination assays were positive at a 1:8 dilution for fractions 21-25 indicating measles virus components were present in those fractions. The densities were also determined and are given in Table 1.

Three separate pools from Figure 9 (shown in brackets) were obtained from fractions 12-15 (pool no. 1), fractions 18-23 (pool no. 2) and fractions 24-28 (pool no. 3). The pools were diluted with TEN

and pelleted at 23,000 RPM for 3 h in a Spinco 30 rotor at 4 C. The pellets were resuspended and centrifuged to equilibrium as described above. Fractions (0.4 ml) were collected and the A_{254} determined (Fig. 10-12).

Fractions 14 and 16 from the gradient profile of pool no. 2 (Fig. 11) were collected and dialyzed against TEN. Electron microscopy of this pool (R. Brooks, Department of Pathology, UOHSC) by negative staining revealed virus-like particles but was insufficient to make any conclusions concerning size distribution. This pool was absorbed onto HeLa cell monolayers undiluted (0.2 ml) or at 1:10 and 1:20 dilutions. Fraction 15 from the same gradient (Fig. 11) was also absorbed onto HeLa cells after being dialyzed. After 24 h and 48 h the cells were fixed with 95% methanol and stained for immunofluorescence. No CPE was observed in these HeLa cells at 37 C during this time period. The 24 h and 48 h HeLa cells treated with undiluted fractions 14 and 16 material were positive for measles virus specific fluorescence. The fluorescence was located exclusively in the cytoplasm of infected cells. Fraction 15 at a 1:20 dilution was also positive at 24 h and 48 h after infection for measles virus fluorescence.

In a subsequent experiment with a concentrated sample of MV_{PI}, purified in a similar manner as described above, the 1.21 g/cc peak fractions were pooled and pelleted in Spinco 30 rotor at 23,000 RPM for 3 h. This pelleted material was then used to adsorb out the measles virus antibody activity from an SSPE serum. The SSPE serum (Davis) was diluted 1:4 over the MV_{PI} pellet and allowed to react for

1 h at 37 C and 12 h at 4 C. The viral material was subsequently pelleted and the serum reacted with $HeLa_{PI}$ cells and studied by indirect immunofluorescence. The results demonstrated that the fluorescence was greatly reduced (>99%) but some fluorescence was still present. It was concluded that because the 1.21 g/cc peak could hemagglutinate Rhesus monkey erythrocytes, could adsorb out or cross-react by immunofluorescence with $HeLa_{PI}$ cells, and since virus-like particles were observed by electron microscopy, that the material in the 1.21 g/cc could contain measles viruses. These particles did not cause CPE in HeLa cells. This is not unusual since the virus was purified directly from the $HeLa_{PI}$ cell cultures where destruction of the cell monolayer would result in loss of the persistence. Further purification and analysis of the biological properties of this 1.21 g/cc peak is necessary to determine its role in the $HeLa_{PI}$ cell system.

References

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Table 1

The density of fractions taken from the gradient in Figure 9.

Fraction no.	Density (g/cc)
13	1.12
14	1.13
20	1.18
21	1.20
22	1.21
25	1.24
28	1.26

Figure 1.

Equilibrium centrifugation of ^{3}H uridine labeled MV_{PI} clone 18. Medium from infected Vero cells labeled with ^{3}H uridine was subjected to low speed centrifugation (10,000 x g for 30 min), pelleted in Spinco 30 rotor for 3 h at 23,000 RPM, resuspended and layered over 15-65% sucrose gradients (12 ml) containing D_2O, and centrifuged at 4 C for 4 h at 39,000 RPM in Spinco SW 41 rotor.

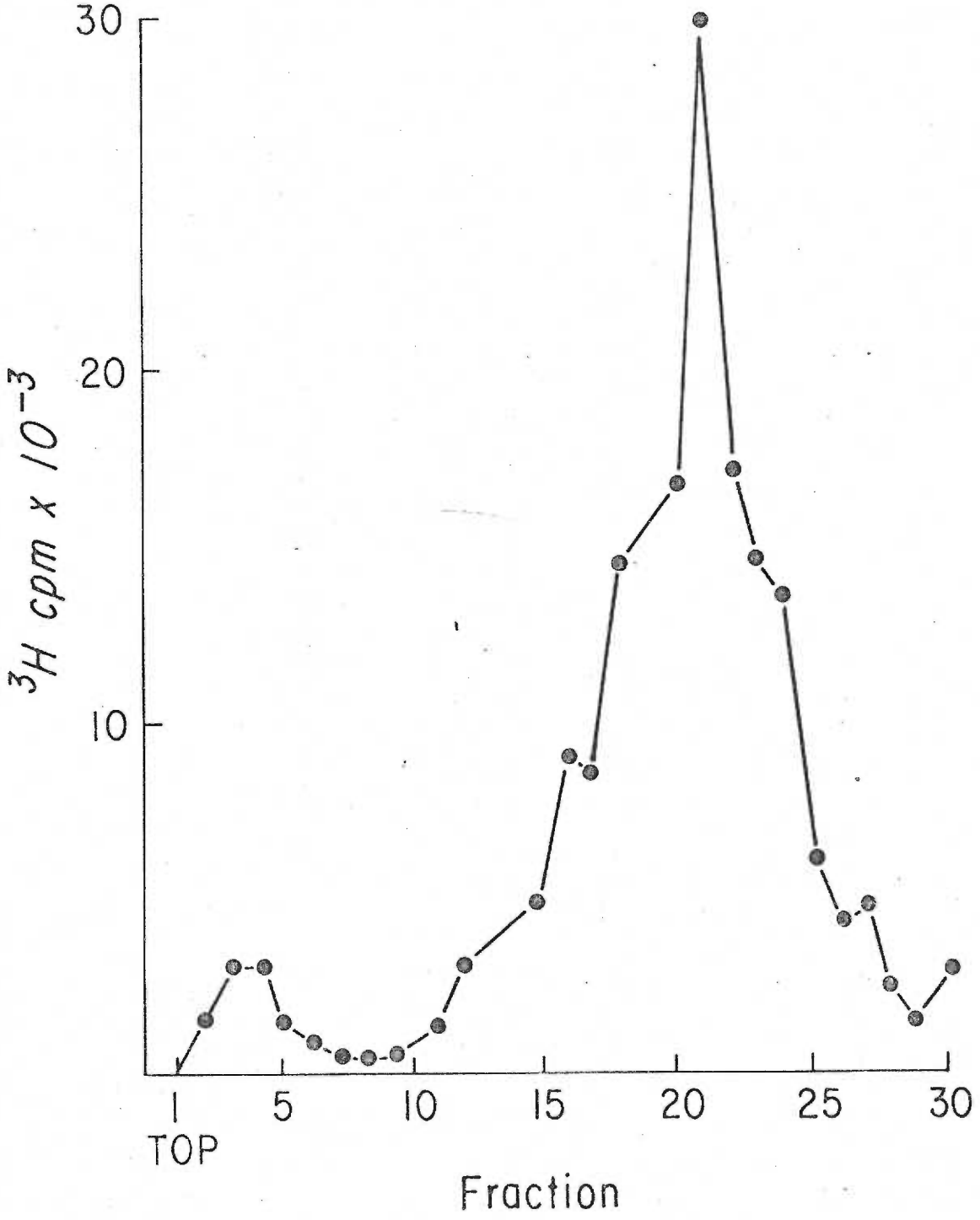

30
20
10
^3H cpm x 10^{-3}
1
TOP
5
10
15
20
25
30
Fraction

Figure 2.

Equilibrium centrifugation of ^{3}H uridine labeled MV_{PI} directly from $HeLa_{PI}$ cells. Cell culture fluid labeled with ^{3}H uridine from $HeLa_{PI}$ cells was processed in a similar manner as described in the legend to Figure 1.

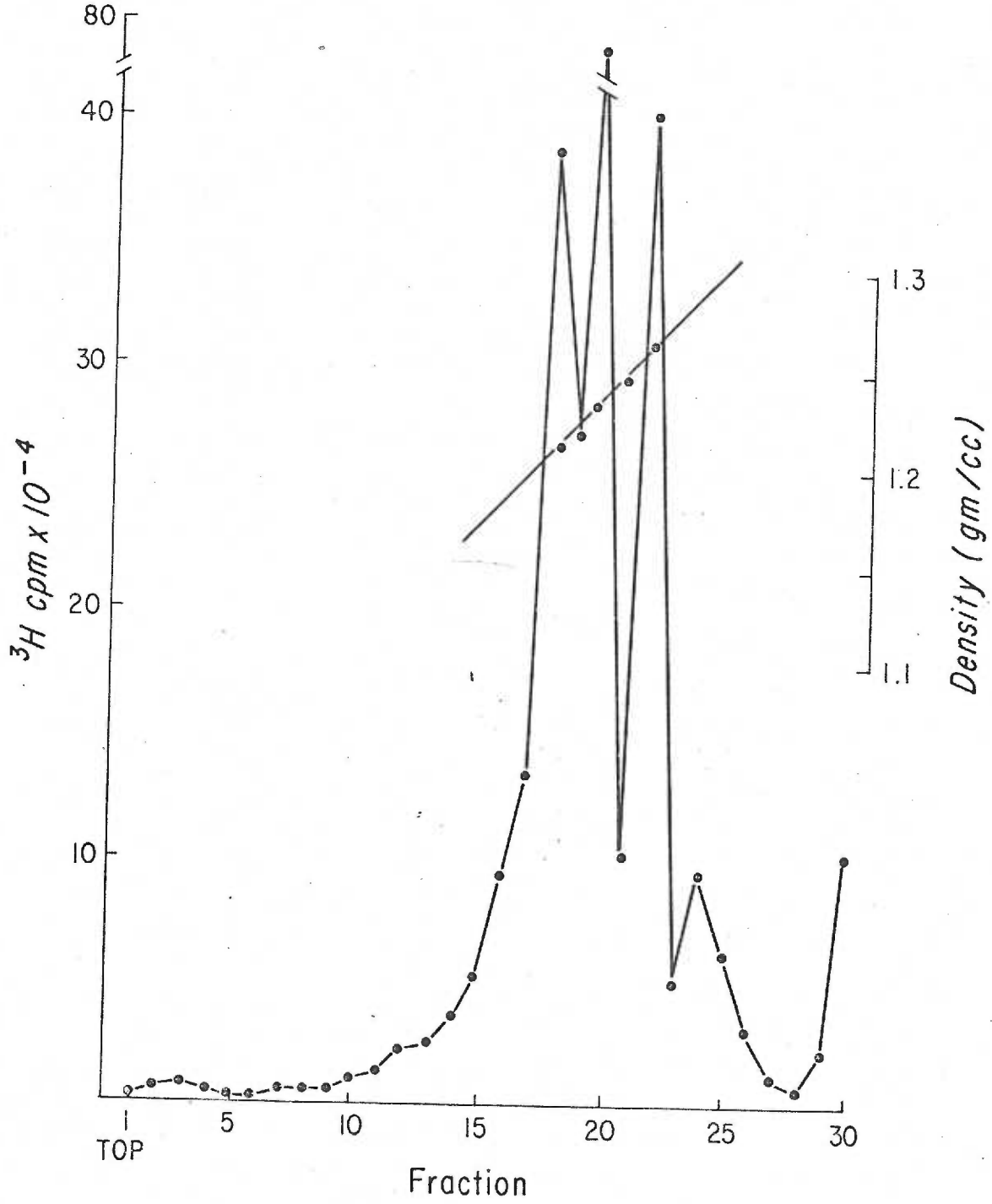

80
40
30
20
10
^3H cpm x 10^{-4}
1.3
1.2
1.1
Density (gm/cc)
1
TOP
5
10
15
20
25
30
Fraction

Figure 3.

Equilibrium centrifugation of ^{3}H uridine labeled wild-type MV_0. Medium from infected HeLa cells labeled with ^{3}H uridine was harvested when cytopathic effect was 4+, centrifuged at low speed to remove cellular debris, concentrated by ultrafiltration at 4 C, and centrifuged for 4 h at 39,000 RPM in Spinco SW 41 rotor in 15-65% sucrose-D_2O gradients.

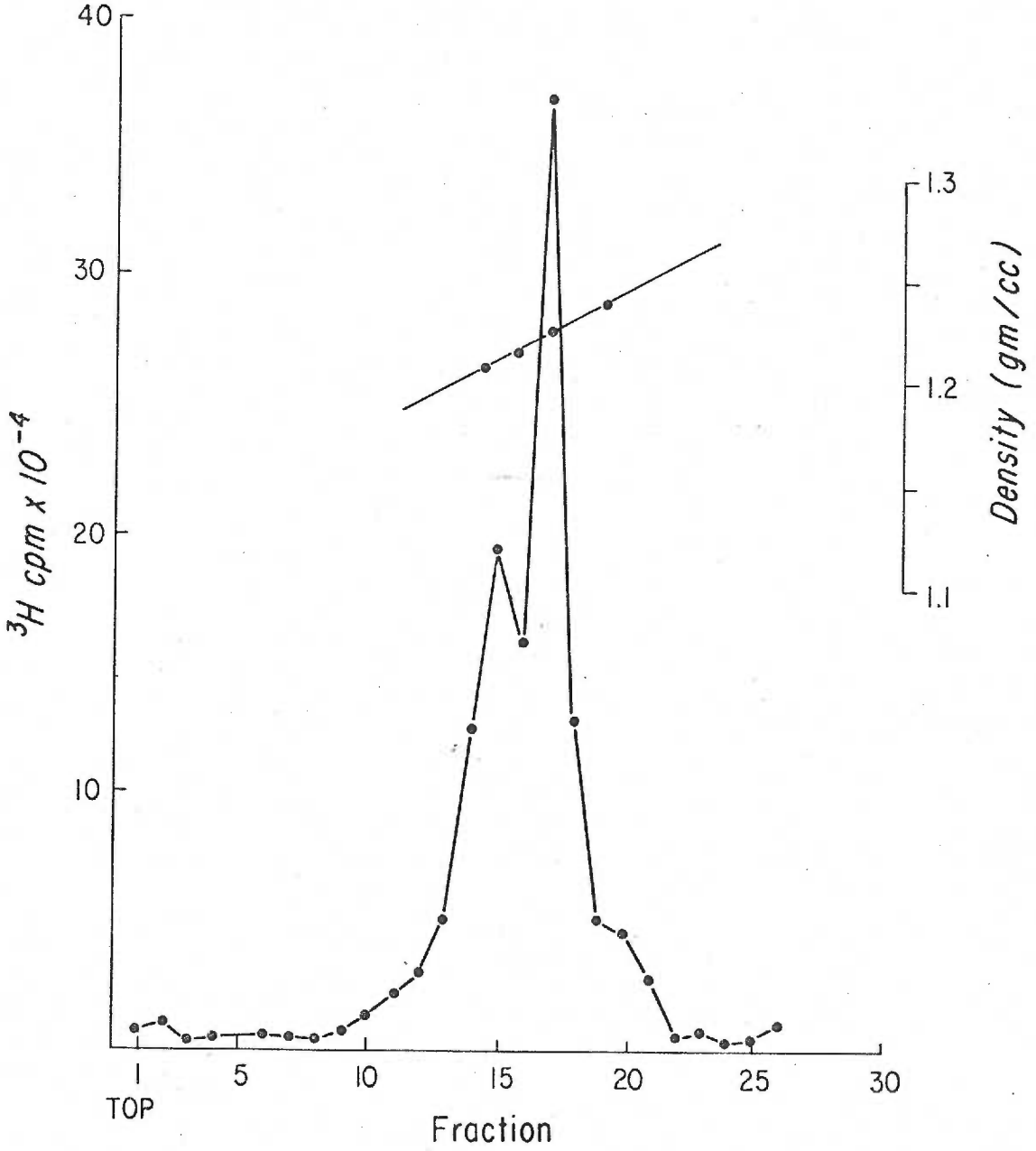

40
30
20
10
^3H cpm x 10^{-4}
1.3
1.2
1.1
Density (gm/cc)
1
TOP
5
10
15
20
25
30
Fraction

Figure 4.

Equilibrium centrifugation of ^{3}H uridine labeled MV_{PI} clone 18 passed one time undilute. The details of the centrifugation are described in the legend to Figure 1.

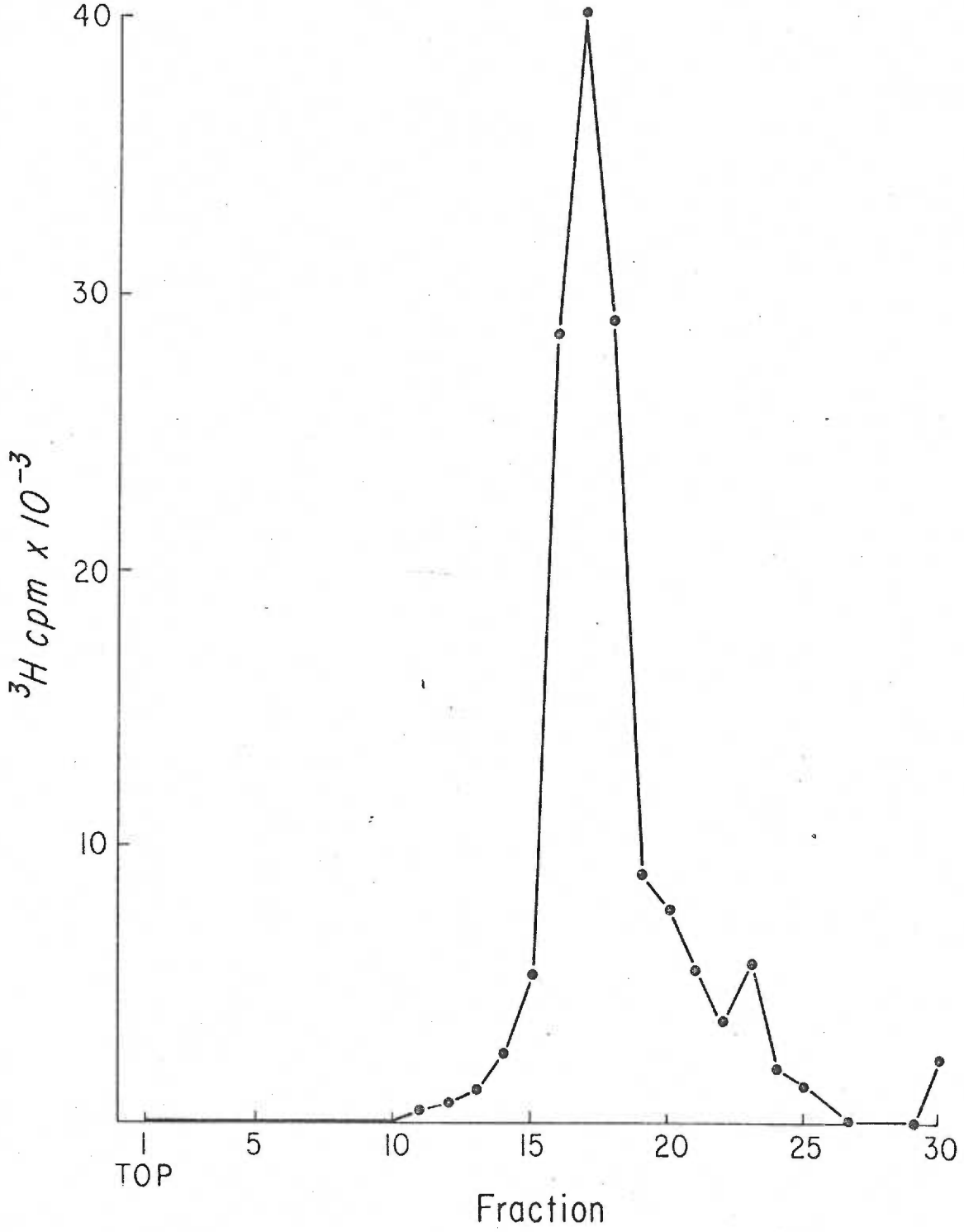

40
30
20
10
^3H cpm x 10^{-3}
1
TOP
5
10
15
20
25
30
Fraction

Figure 5.

Equilibrium centrifugation of ^{3}H uridine labeled MV_{PI} clone 18 passed two times undilute. The details of the centrifugation are described in the legend to Figure 1.

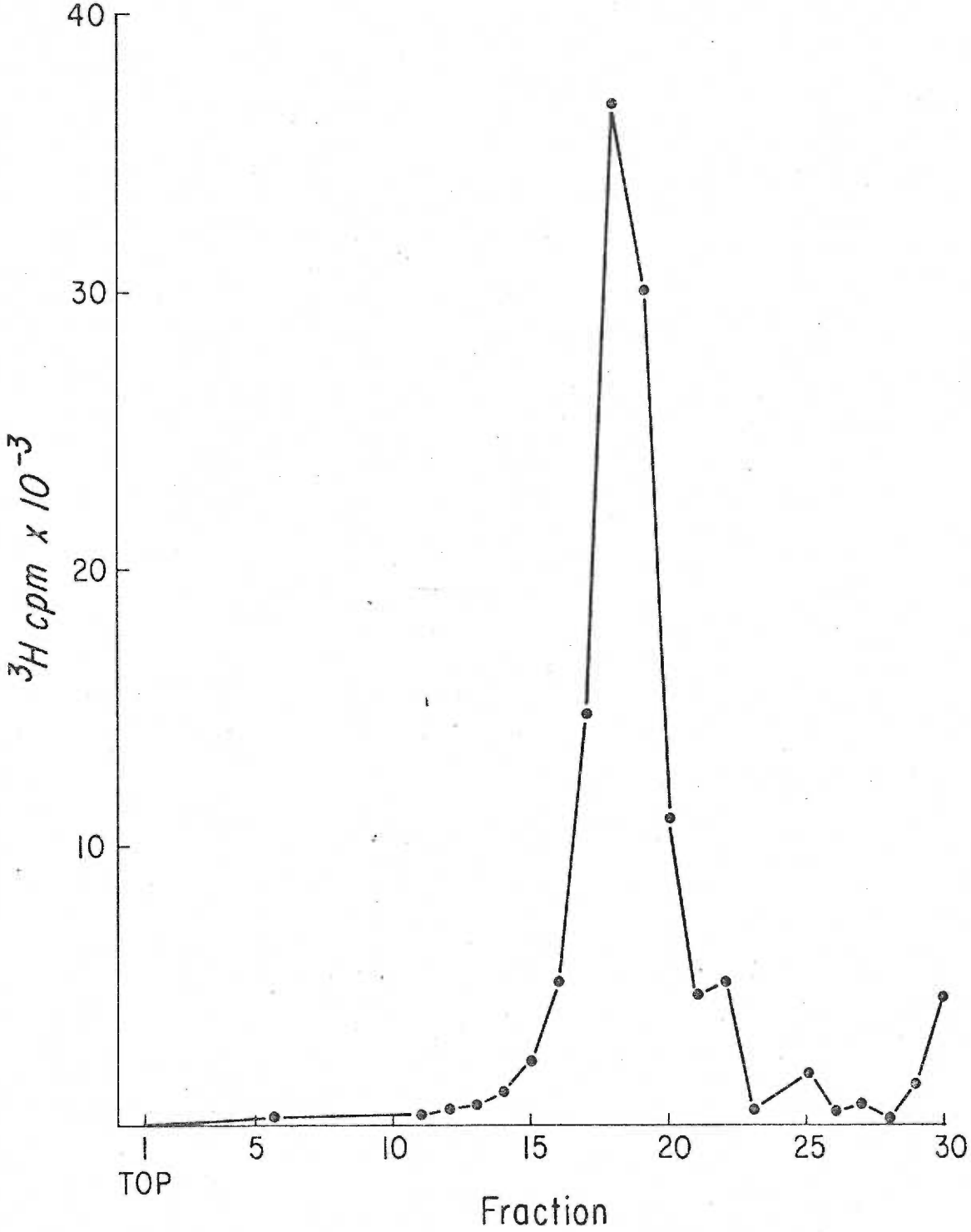

40
30
20
10
^3H cpm x 10^{-3}
1
TOP
5
10
15
20
25
30
Fraction

Figure 6.

Equilibrium centrifugation of ^{3}H uridine labeled MV_{PI} clone 18 passed undiluted three times. The details of the centrifugation are described in the legend to Figure 1.

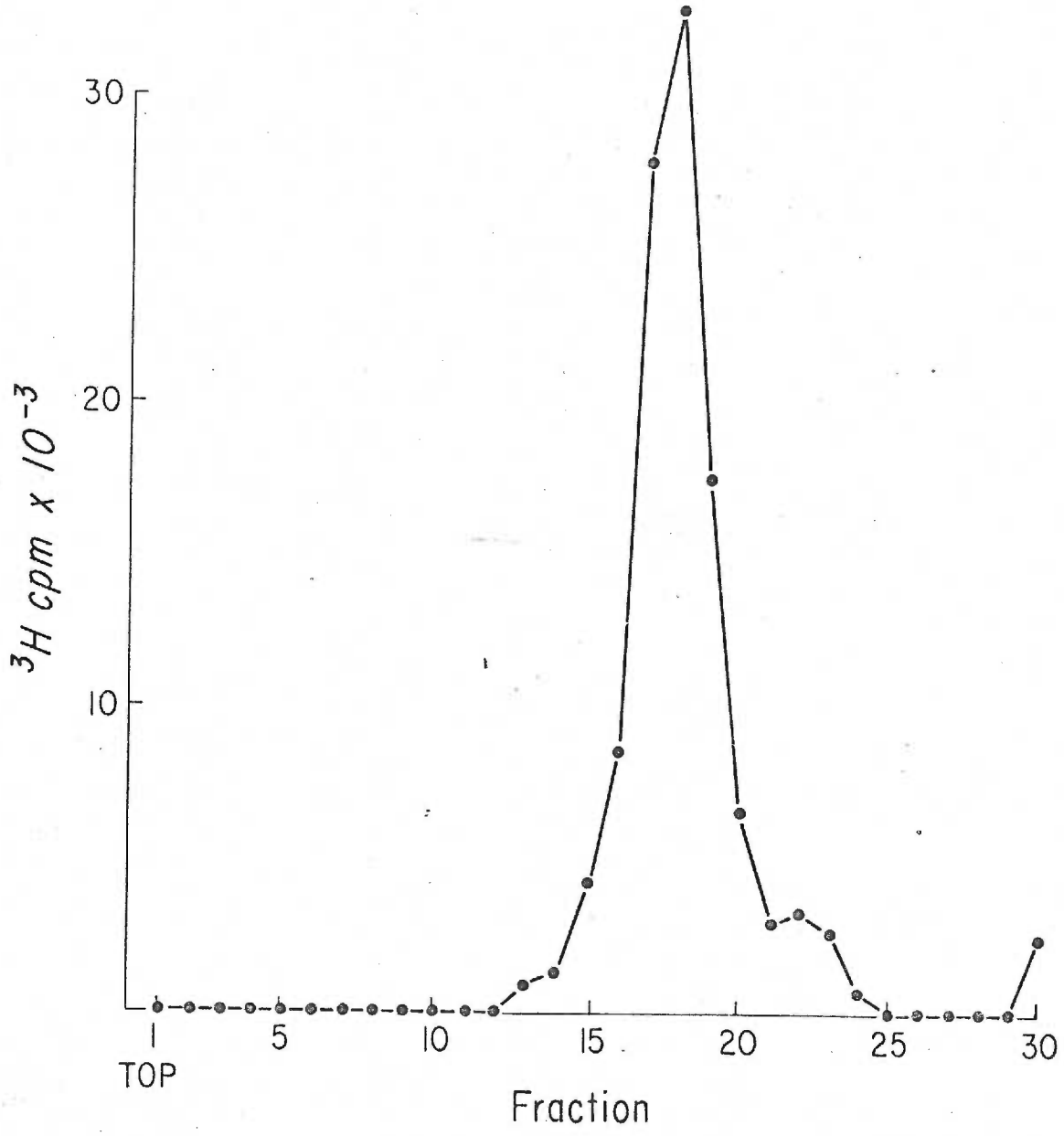

30
20
10
^3H cpm x 10^{-3}
1
TOP
5
10
15
20
25
30
Fraction

Figure 7.

Equilibrium centrifugation of ^{3}H uridine labeled MV_{PI} clone 18 passed undiluted four times (no sonication). The details of the centrifugation are given in the text and in the legend to Figure 1.

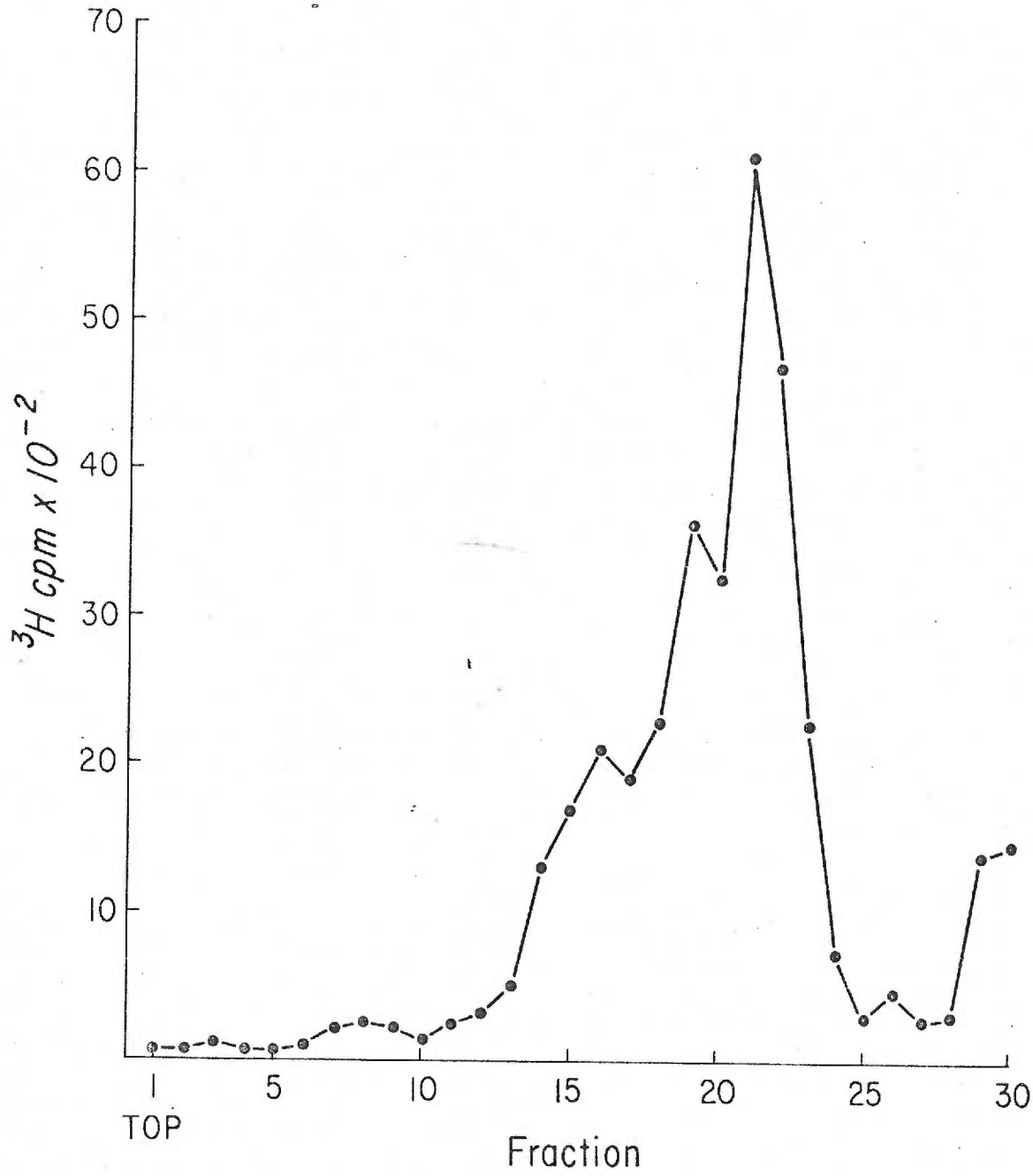

70
60
50
40
30
20
10
^3H cpm x 10^{-2}
1
TOP
5
10
15
20
25
30
Fraction

Figure 8.

Equilibrium centrifugation of ^{3}H uridine labeled MV_{PI} clone 18 passed undiluted four times (sonication). The details of the centrifugation are given in the text and in the legend to Figure 1.

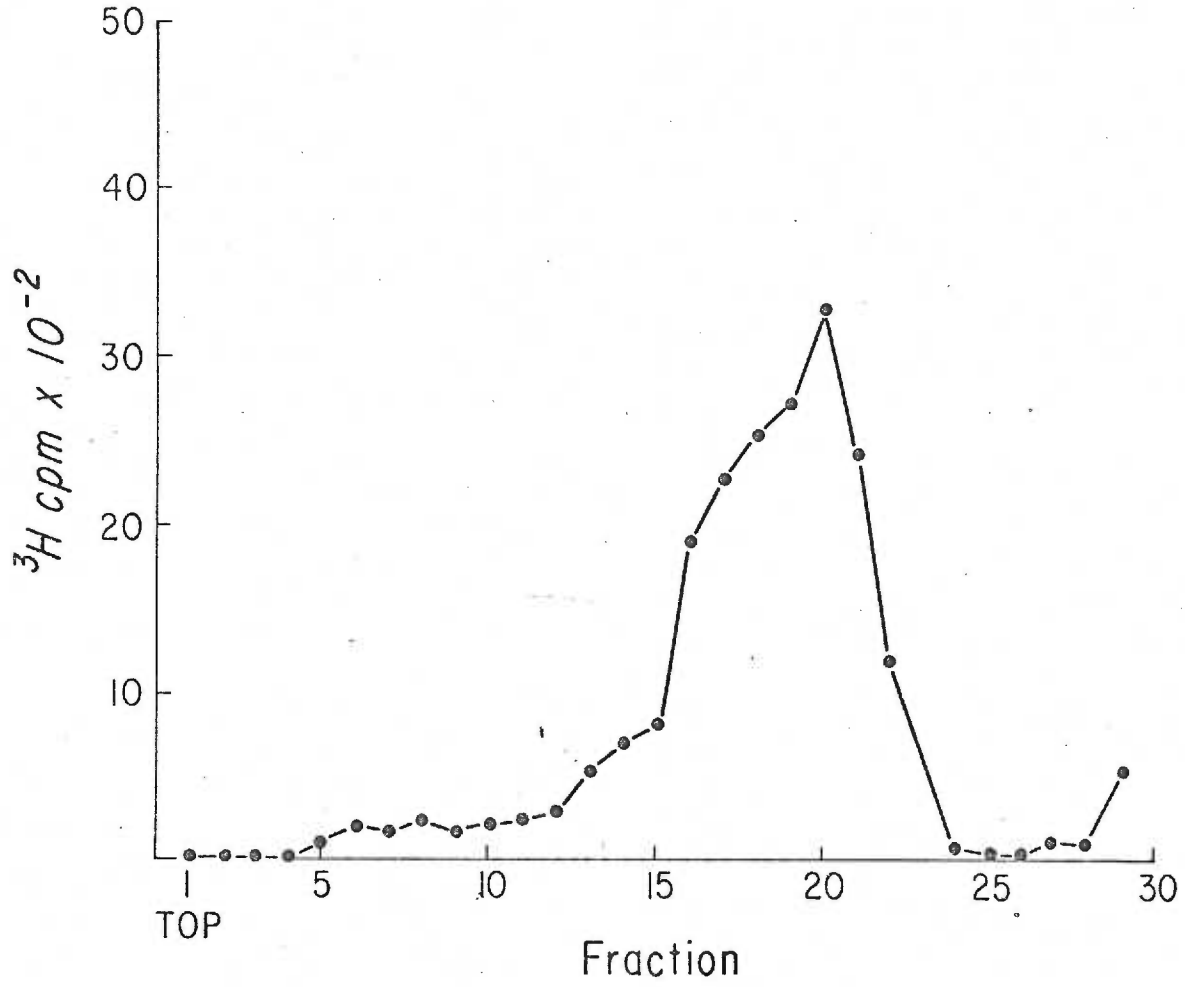

50
40
30
20
10
^3H cpm x 10^{-2}
1
TOP
5
10
15
20
25
30
Fraction

Figure 9.

Equilibrium centrifugation of MV_{PI} taken from $HeLa_{PI}$ cells. $HeLa_{PI}$ cell fluids were clarified by low speed centrifugation (10,000 x g for 30 min), pelleted in a Spinco 30 rotor for 3 h at 23,000 RPM, resuspended and layered over a 15-65% sucrose-D_2O gradient, and centrifuged at 4 C for 12 h at 23,000 RPM in a Spinco SW 27 rotor.

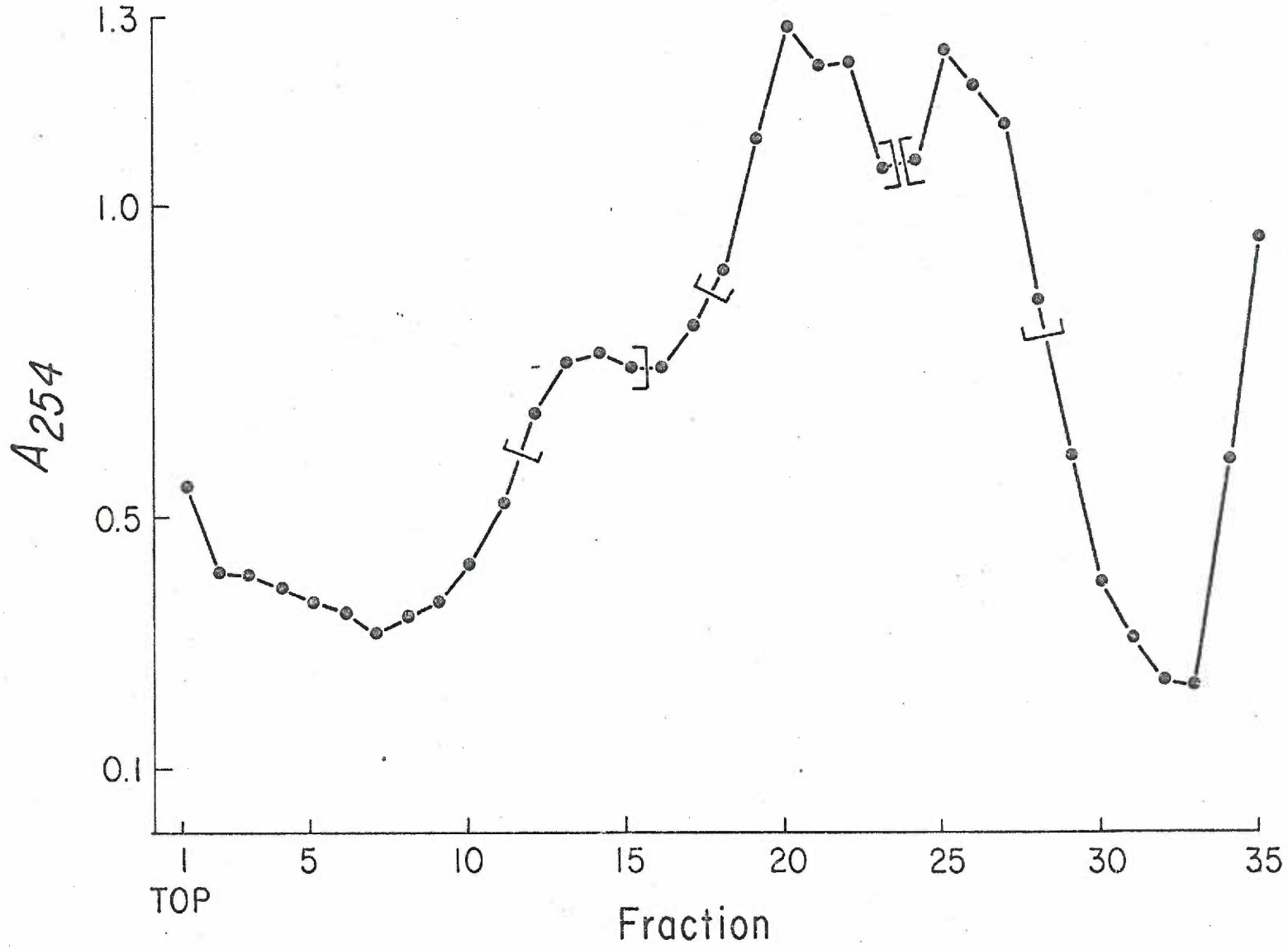

1.3
1.0
0.5
0.1
A254
1
TOP
5
10
15
20
25
30
35
Fraction

Figure 10.

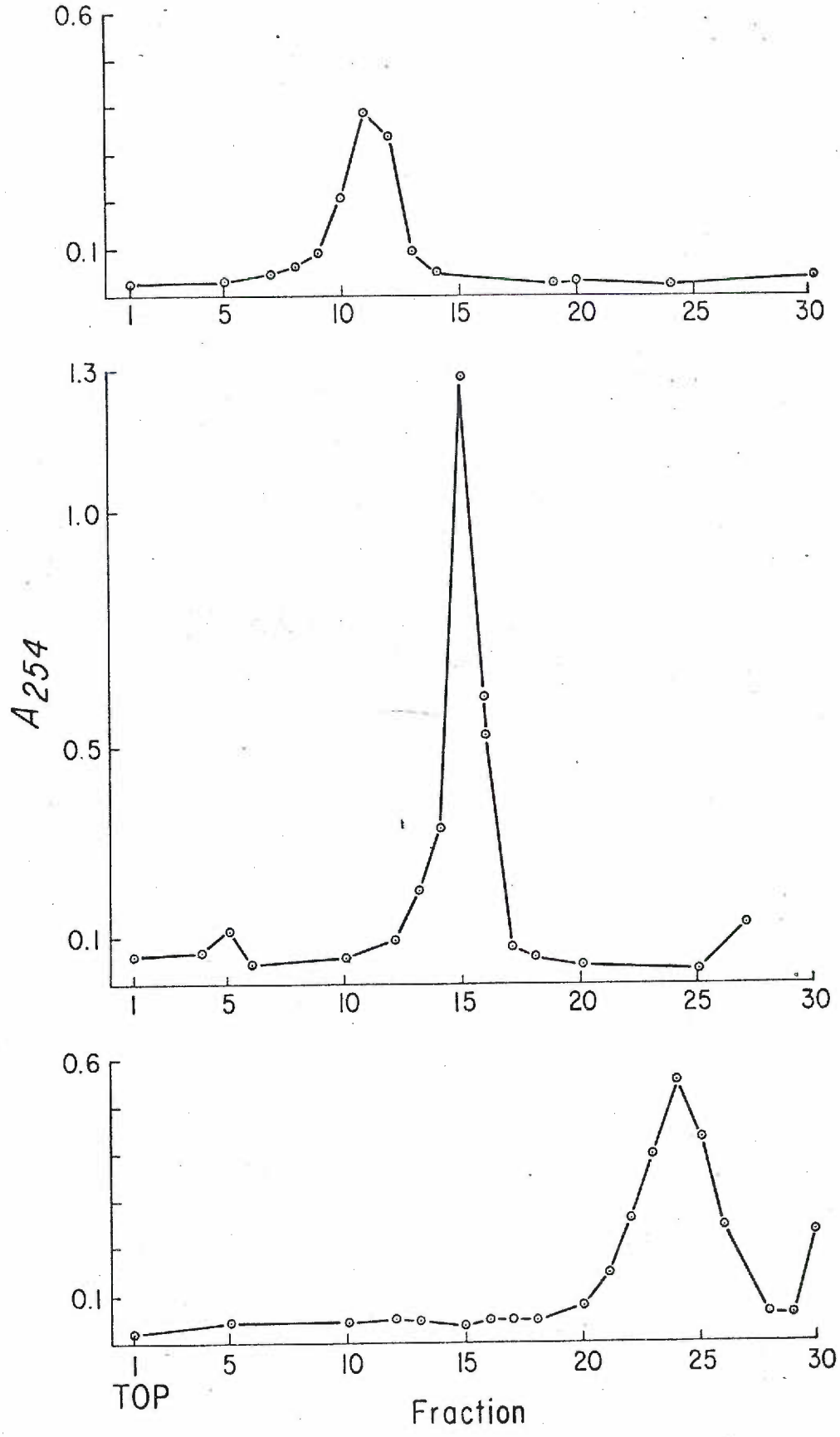
Equilibrium centrifugation of pool no. 1 from Figure 9. Fractions 12-15 were diluted with TEN buffer, pelleted at 23,000 RPM for 3 h in a Spinco 30 rotor at 4 C, resuspended and centrifuged in a Spinco SW 41 rotor for 4 h at 39,000 RPM at 4 C.

Figure 11.

Equilibrium centrifugation of pool no. 2 from Figure 9. Fractions 18-23 were diluted with TEN buffer and processed as described in the legend to Figure 10.

Figure 12.

Equilibrium centrifugation of pool no. 3 from Figure 9. Fractions 24-28 were processed as described in the legend to Figure 10.


0.6
0.1
1 5 10 15 20 25 30
1.3
1.0
0.5
0.1
A254
1 5 10 15 20 25 30
0.6
0.1
1 5 10 15 20 25 30
TOP
Fraction

VI. LIST OF ABBREVIATIONS

A	- adenine
ATV	- alkaline trypsin versene
C	- centigrade or cytosine
^{14}C	- carbon 14
CDV	- canine distemper virus
CF	- complement fixing
CPE	- cytopathic effect
CsCl	- cesium chloride
DIP	- defective interfering particles
DNA	- deoxyribonucleic acid
EDTA	- ethylenedinitrilotetraacetic acid
EOP	- efficiency of plaquing
FCS	- fetal calf serum
G	- guanine
g	- gravity or grams
g/cc	- grams/cubic centimeter
g/l	- grams/liter
h	- hour
^{3}H	- tritium
HA	- hemagglutination
HAD	- hemadsorption
$HeLa_{PI}$ cells	- HeLa cells persistently infected with measles virus
HL	- hemolytic
IF	- immunofluorescence

log_{10}	- logarithm base 10
ME	- mercaptoethanol
MEM	- minimal essential medium
min	- minute
ml	- milliliter
mm	- millimeter
Mn	- manganese
moi	- multiplicity of infection
MV	- measles virus
MV_o	- wild-type measles virus
MV_{PI}	- measles virus from persistently infected HeLa cells
MW	- molecular weight
NaCl	- sodium chloride
NDV	- Newcastle disease virus
nm	- nanometer
PAGE	- polyacrylamide gel electrophoresis
PBS	- phosphate buffered saline
PFU/ml	- plaque forming units/milliliter
RNA	- ribonucleic acid
RPM	- revolutions per minute
S	- Svedberg unit (sedimentation rate)
SDS	- sodium dodecyl sulfate (sodium lauryl sulfate)
SSC	- standard saline citrate
SSPE	- subacute sclerosing panencephalitis
SV-5	- Simian virus 5
$TCID_{50}$	- tissue culture infective dose $_{50}$

ts	- temperature sensitive
tsT	- temperature sensitive mutant T
U	- uracil
µ	- micron
µCi/ml	- microCurie/milliliter
µg	- micrograms
U	- units
VSV	- vesicular stomatitis virus

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