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Activation of a Latent Measles Virus Infection in Hamster Cells

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The characteristics of infectious measles virus released from latently infected hamster embryo fibroblast cells are described. Low levels of virus were released spontaneously when the cultures were incubated at 37 C; this phenomenon was observed 19 passages after the cells had been exposed to the virus and has continued through cell passage 45. The virus yield could be significantly increased by cocultivation of the hamster cells with BSC-1 cells or incubation of the latently infected cells at 33.5 C rather than at 37 C. Measles virus released after cocultivation demonstrated increased cytopathology in cell culture and reduced temperature sensitivity when compared to the virus released at 33.5 C. After cell passage 45, there was an increase in spontaneous release of virus. However, the viruses recovered by cocultivation or temperature release after cell passage 45 were nearly identical. These observations suggest a possible mechanism for measles virus activation in cells latently infected with this virus.

Latent infection with measles virus has been postulated as a prerequisite for the pathological effects observed in several neurological diseases. Measles virus has been recovered from biopsies from the central nervous system (3) taken from cases of subacute sclerosing panencephalitis (SSPE). The virus has also been implicated as one of the possible etiological agents of multiple sclerosis by serological techniques (2).

Several investigators have described latent infections in vitro due to measles virus (4, 5, 11). In these experiments, virus could be induced by incubation of the cells at 33 C, rather than incubation at 37 C. Recently, it has been demonstrated that an in vivo latent infection of newborn hamsters could be established when the animals possessed high titers of maternally acquired measles neutralizing antibody prior to exposure to virus (12). Synthesis of measles virus could subsequently be activated by treatment of the animals with cyclophosphamide. A latent infection in vitro involving Schwarz measles virus in hamster embryo fibroblast (HEF) cells has also been described (4). The block in virus replication which resulted in virus latency occurred late in the infectious cycle, after virus RNA and protein synthesis. This report describes the activation of infectious measles virus from the latently infected HEF cells and presents a possible mechanism for virus activation involving proliferation of the latently infected cells.

MATERIALS AND METHODS

Virus. The Schwarz vaccine strain of measles virus was obtained commercially from Dow Chemical Corp. Virus stocks had been passaged eight times in BSC-1 cells prior to use in these experiments. Cell monolayers in 8-oz (approximately 0.224 kg) glass bottles were infected with measles virus, and the virus was harvested 96 h after infection by scraping the cells from the bottle surface. The virus-cell suspension was treated by sonic oscillation (45 s) and was quick frozen in a dry ice-alcohol bath. All measles virus stocks were stored at −76 C in a Kelvinator low temperature freezer.

Cells. HEF cells were prepared by trypsinization of 13 day-old decapitated Syrian hamster embryos from a single litter (1). All hamster cells were grown at 37 C in 8-oz glass prescription bottles containing medium 199 supplemented with 10% fetal calf serum, 10% tryptose phosphate broth, and 0.08% $NaHCO_3$. The derivation and characterization of the HEF cells latently infected with measles virus (S cells) has been previously described (4). The first-phase S cells (S-1) were the latently infected HEF cells from passage 1 through passage 18, second-phase S cells (S-2) were in passage 19 through passage 44, and the third-phase S cells (S-3) were the latently infected cells after cell culture passage 45. BSC-1 cells were obtained from R. Dulbecco, Salk Institute and were grown in Eagle basal medium (EBM) containing 10% fetal calf se-

rum, 10% tryptose phosphate broth, and 0.12% $NaHCO_3$. After infection with measles virus, EBM was also supplemented with 1 mM L-arginine (10).

Cocultivation. The method of measles virus rescue by cocultivation from latently infected hamster cells has been previously described (4). Briefly, a suspension of approximately 10^6 BSC-1 cells was added to an 8-oz bottle containing about 10^6 attached S cells. The cell mixture was harvested 20 h after the addition of the BSC-1 cells and assayed for infectious measles virus. The experimental temperatures (33.5, 37, and 39 C) for cocultivation, temperature-induction, and growth studies were maintained within a temperature variation of ± 0.5 C.

Heat inactivation. Five milliliters of the appropriate measles virus stock was placed at 41.5 C. At 0, 10, and 40 min, 1-ml samples were removed. The 1-ml samples were then stored at 4 C until all had been collected, at which time they were quick frozen in a dry ice-alcohol bath, and stored at −76 C until assayed for infectious measles virus in BSC-1 cells by the plaque technique.

Virus plaque assay. Prior to cell adsorption virus samples were briefly sonically treated (15 s). BSC-1 cell monolayers, grown in 60-mm Falcon plastic petri dishes, were inoculated with 0.1 ml of the measles virus suspension to be assayed. The virus inoculum was diluted with 0.4 ml of Tris-buffered saline (pH 7.4, 0.025 M) after addition to the cultures to prevent drying of the cells. After virus adsorption for 2 h at room temperature, the BSC-1 cell monolayers were overlaid with EBM containing 1 mM L-arginine, 10% fetal calf serum, 1% agar, and 0.23% $NaHCO_3$. The infected cultures were kept in a 5% CO_2 incubator at 33.5 or 37 C for 7 days, after which time 2 ml of Tris-buffered saline (pH 7.4) containing a 1:20,000 dilution of neutral red was added. Plaques were counted 8 days after initial virus adsorption, and titers are presented as PFU per milliliter.

RESULTS

Release of measles virus from latently infected hamster cells. Latently infected HEF cells (S-2) could be induced to release infectious measles virus after incubation of the cells at 33.5 C or by cocultivation with BSC-1 cells (Table 1). The maximum release of virus (3×10^4 PFU/ml) through temperature induction occurred at 72 h at 33.5 C, whereas maximum yields (1.4×10^5 PFU/ml) after cocultivation with BSC-1 cells were obtained at 20 h at 39 C. Infectious measles virus was also released at all temperatures tested after cocultivation with BSC-1 cells (Table 1). However, there was an inverse relationship in the quantities released by temperature induction when compared to release at various temperatures after cocultivation of cells. No detectable virus was spontaneously released at 39 C, whereas this temperature resulted in maximal virus yields after cocultivation. Maximal yields of measles virus was released without cocultivation at 33.5 C. Significantly less virus was released in the absence of BSC-1 cells when the incubation temperature was 37 C than was released when the hamster cells were cocultivated with the BSC-1 cells and incubated at 37 C.

Properties of the virus released from S-2 cells. The virus obtained after temperature-release (TR) differed in certain properties from the virus obtained after cocultivation. Replication of the TR virus was temperature sensitive (Fig. 1), and no virus replication was detectable at 39 C until more than 60 h after infection of BSC-1 cells. At 84 h postinfection, there was 3.5 log difference in the titer of TR virus grown at 33.5 and at 39 C. Although the cocultivation (CC)-released virus replicated better at 33.5 than at 39 C, it does not differ from the parental Schwarz virus in this respect (Fig. 1). Inactivation of both CC and TR viruses yielded similar results at 41.5 C (Table 2). The temperature sensitivity of the TR virus is therefore probably not due to a decrease in the stability of the virion to heat.

There was also a consistent difference in plaque size induced by the two virus types. Large plaques (≥1 mm in diameter) were typical of the CC-released virus, and small plaques (≤0.5 mm in diameter) were characteristic of the TR virus. This is in agreement with the observation that TR virus grows to lower titers than the CC-released virus. However, temperature sensitivity and plaque size were found to be independent factors as nontemperature sensitive, small plaque-producing TR virus variants could be readily isolated.

TABLE 1. *Induction of measles virus from second phase (S-2) cells*[a]

Method of virus release	Incubation temperature		
	33.5 C	37 C	39 C
Cocultivation[b]	2.5×10^4 [c]	2.4×10^4	1.4×10^5
Temperature released[d]	3.0×10^4	4.0×10^2	<10

[a] S-2 cells during cell culture passage number 35. Similar results were obtained whenever S-2 cells were tested from cell passage 19 through 44.

[b] Introduction of 10^6 BSC-1 cells into an 8-oz (approximately 0.224 kg) prescription bottle containing 10^6 attached S-2 cells. Cells were harvested 20 h after the addition of the BSC-1 cells.

[c] PFU of measles virus per milliliter assayed in BSC-1 cells.

[d] Cells maintained at the indicated temperatures were harvested after 72 h of incubation at that temperature.

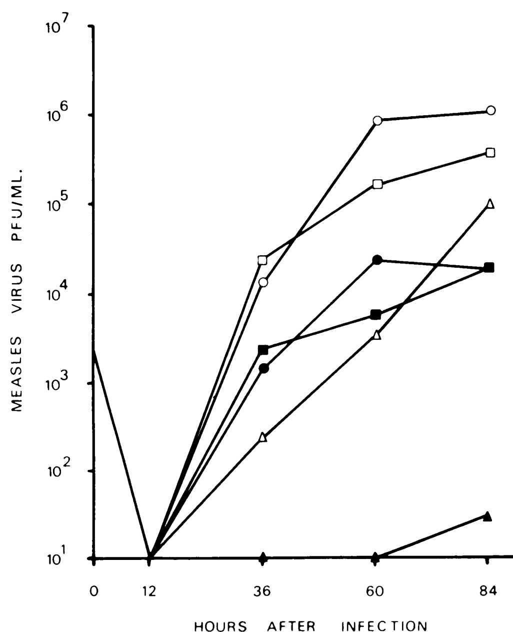

FIG. 1. *Growth of early system (S-2) cocultivation (CC)-released viruses, temperature-released (TR) viruses, and Schwarz measles virus in BSC-1 cells at 33.5 and 39 C. Symbols: ▲, TR 39 C; △, TR 33.5 C; ●, CC 39 C; ○, CC 33.5 C; ■, Schwarz 39 C; and □, Schwarz 33.5 C. Measles virus was assayed in BSC-1 cells by the plaque technique.*

There was a delay in release of infectious TR virus grown in Vero cells when compared with the CC-released virus (Fig. 2). No TR virus was detectable at 36 h postinfection, while the CC-released virus reached titers of 10^3 PFU/ml after the same time interval. The TR virus from S-2 cells, therefore, appeared to be less adaptable for growth in Vero cells than the CC-released virus. It should be noted that the eclipse phase of measles virus in Vero cells was 16 h; it is 24 h in BSC-1 cells.

Schwarz vaccine virus produces a latent infection in nonproliferating HEF cells (4); a lytic infection is, however, produced in actively proliferating HEF cells (P. Knight, unpublished observation). To further study the effect of cell proliferation upon the nature of the virus infection, as well as to determine whether the CC virus was more cytopathic for HEF cells than the parental Schwarz virus, actively proliferating and static phase HEF cells were infected with these viruses. The CC virus released from the S-2 cells replicated only slightly more efficiently in hamster cells than the parental Schwarz vaccine virus (Table 3). Peak titers of approximately 2×10^3 PFU/ml were obtained in nonproliferating HEF, utilizing the CC-released virus as well as the Schwarz vaccine virus that was used to originally establish the latent infection. However, measles virus replicated more efficiently in actively proliferating HEF cells than in static phase cultures. Titers of about 5×10^4 PFU/ml were obtained (Table 3). Again, the CC virus and Schwarz virus gave similar results.

Properties of virus released from S-3 cells. Both the CC and TR viruses isolated from a late passage or third phase (S-3) of the cells had properties which were different than the measles virus released from the S-2 cells. The TR virus was no longer temperature sensitive at 39 C; replication proceeded equally well at 33.5

TABLE 2. *Heat inactivation of cocultivation-released and temperature-released measles virus*[a]

Inactivation[b] (min)	Cocultivation-released virus		Temperature-released virus	
	PFU/ml[c]	Survivors (%)	PFU/ml	Survivors (%)
0	3.3×10^6	100	5.5×10^5	100
10	2.2×10^6	67	4.2×10^5	76
40	7.9×10^5	24	1.0×10^5	18

[a] See Materials and Methods for details of temperature inactivation procedures.

[b] Virus was inactivated while in suspension in a 41.5 C water bath.

[c] PFU of measles virus per milliliter assayed in BSC-1 cells.

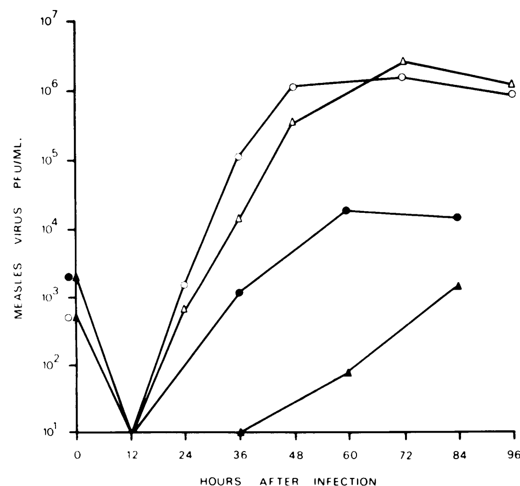

FIG. 2. *Growth of cocultivation-released (CC) and temperature-released measles (TR) viruses in Vero cells at 37 C. Symbols: ●, early system CC, S-2; ▲, early system, S-2 TR; ○, late system CC, S-3; and △, late system TR, S-3. The released virus was assayed in BSC-1 cells by the plaque technique.*

TABLE 3. *Growth of Schwarz measles virus and cocultivation released virus in proliferating and nonproliferating hamster embryo fibroblast cells*

Day	Schwarz virus[a]		Cocultivation virus[b]	
	Static HEF[c]	Proliferating HEF[d]	Static HEF	Proliferating HEF
1[e]	<10[f]	<10	<10	<10
2	<10	<10	<10	<10
3	<10	<10	<10	<10
4	<10	<10	6.0×10^1	7.0×10^1
5	3.0×10^1	3.7×10^2	1.8×10^3	1.6×10^3
10	1.1×10^3	3.4×10^3	1.8×10^3	3.4×10^4
15	1.5×10^3	5.1×10^4	3.8×10^2	5.2×10^4
20	6.3×10^2	2.4×10^3	1.6×10^3	1.8×10^3
25	3.5×10^2	1.3×10^2	2.7×10^2	8.0×10^1
30	2.5×10^2	5.0×10^1	1.1×10^1	<10

[a] HEF cells were infected with an input multiplicity of infection (MOI) of 0.05 PFU of measles virus per cell.
[b] HEF cells were infected with an MOI of 0.05 PFU of cocultivation-released measles virus from passage 35 S-2 cells.
[c] HEF cells were infected as a monolayer. On day 1 after infection the cells were diluted 1:2, passed, and maintained for the remainder of the study as a confluent monolayer.
[d] Cells were infected as a monolayer. On day 1 after infection they were passed from 1 bottle into 2 bottles. During the course of the study the cultures were inspected daily, and when 100% confluency was attained, the cultures were passed from 1 bottle into 2 bottles.
[e] Days after measles virus infection.
[f] PFU of measles virus per milliliter assayed in BSC-1 cells.

and 39 C (Fig. 3). Both the CC and TR viruses grew to higher titers (up to 10^7 PFU/ml) than did the viruses isolated from the earlier passage of the S cells (10^4–10^6 PFU/ml). The plaques produced by the TR virus were large (≥1 mm in diameter) and were indistinguishable from the plaques produced by the CC-released virus. TR virus no longer exhibited the characteristic growth lag when first grown in Vero cells (Fig. 2). Both CC and TR viruses were detectable 24 h postinfection. The late-system CC and TR viruses also grew to higher titers in Vero cells than did the viruses induced from the S-2 cells. As observed with the early system (S-2) CC virus, the S-3 CC and TR viruses did not replicate more efficiently in hamster cells than did the parental Schwarz virus. Over the course of 5 days in stationary cultures of HEF, peak titers of 3×10^1 PFU/ml were obtained with both the S-3 CC and TR viruses at 33.5 and 39 C.

An increase in the release of virus was observed when the S-3 cells were incubated at 33.5 C (Table 4). Therefore, the TR virus was still present and capable of activation but possessed properties indistinguishable from those of the CC released virus. Unlike the early system cells, titers of 6×10^2 PFU/ml were released spontaneously by the S-3 cells at 39 C, indicating an apparent breakdown of the latent infection.

The release of measles virus from S-3 cells was also dependent upon the length of time after passage of the cell culture (Fig. 4). Significantly more infectious virus was released per cell when cultures were tested for virus production at 20 and 44 h after cell passage. As the cultures approached confluency, the yield of infectious measles virus per cell was significantly reduced. These results can be interpreted as further evidence of the requirement for cell proliferation during the activation of latent measles virus in this system.

DISCUSSION

Measles virus can enter a latent state in nonproliferating HEF cells. When the latently infected cells are allowed to proliferate, the infection progresses, after many cell passages, into an overt infection. Apparently, the cells pass through at least three phases during activation of the latent infection.

During the first phase, as we have previously reported (4), no detectable virus is spontaneously released from the cells. Virus (>10^5 PFU/ml), however, could be rescued by cocultivation with BSC-1 cells. This first phase, involving true latency, lasted through passage 18.

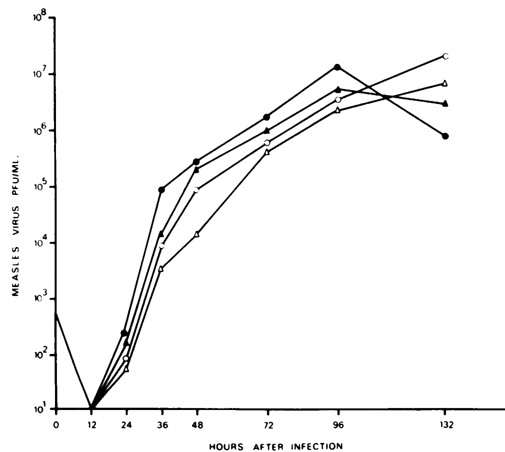

FIG. 3. *Growth of late system (S-3) cocultivation (CC)- and temperature-released (TR) measles viruses in BSC-1 at 33.5 and 39 C. Symbols:* ○, *CC 33.5 C;* ●, *CC 39 C;* △, *TR 33.5 C; and* ▲, *TR 39 C. The induced measles virus was assayed in BSC-1 cells by the plaque technique.*

TABLE 4. *Induction of virus from S-3 cells*[a]

Method of virus induction	Temperature		
	33.5 C	37 C	39 C
Cocultivation[b]	1.8×10^{5}[c]	1.4×10^{6}	1.6×10^{6}[d]
Temperature release[e]	8.0×10^{4}	9.5×10^{2}	6.2×10^{2}

[a] S-3 cells during passage 45.

[b] BSC-1 cells (10^{6}) were added to an 8-oz (approximately 0.224 kg) prescription bottle containing 10^{6} attached S-3 cells and harvested 20 h after the addition of BSC-1 cells.

[c] PFU per milliliter assayed in BSC-1 cells.

[d] After 20 h, measles virus CPE involved most of the cells and caused cellular disintegration. Therefore, the peak titer was reached before 20 h, and heat inactivation probably resulted in a reduction of the titer obtained at 20 h.

[e] Cells harvested 72 h after cell passage and grown at the appropriate temperature.

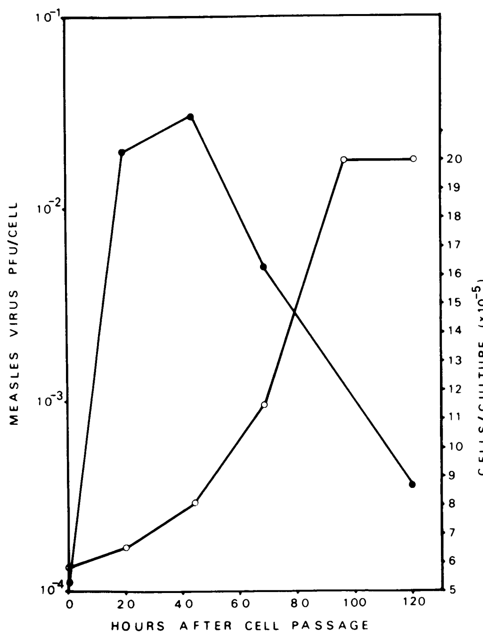

FIG. 4. *Increased production of measles virus in late system (S-3) cultures after cell passage. Latently infected cultures were passed by trypsin dispersion of cell monolayers and multiple replicate cultures were grown in 60-mm plastic petri dishes. Three cultures were harvested at 0, 20, 44, 68, and 120 h after cell passage and assayed for infectious measles virus by the plaque method in BSC-1 cells. Three additional replicate cultures were also trypsinized at 0, 20, 44, 68, 96, and 120 h after cell passage, and the total number of cells on each plate was determined. Results are presented as the average number of infectious measles virus particles per cell (●) and as the average number of cells per 60-mm petri dish (○).*

Cell passage 19 marked the transition point between the first and second phases. During the second phase, limited amounts of virus were spontaneously released at 37 C. A two log increase in the release of virus could be obtained by incubation of the cells at 33.5 C. No detectable virus was released at 39 C. The virus was temperature sensitive if grown at 39 C, and, in general, less virulent (9) (small plaque size, lower titers and less adaptable to other cell lines) than the CC-released virus. There are some similarities between our system and the Newcastle disease virus (NDV)-L system described by Preble and Youngner (8). The virus released in both systems is temperature sensitive, produces small plaques, and demonstrates independence between plaque size and temperature sensitivity. In addition, the temperature sensitivity is not due to a decrease in the heat stability of the virion. Unlike the NDV-L cell system, which became more virulent in L cells, the virus released from our system was able to undergo only a slightly more productive infection in HEF cells than the parental virus. In addition, the NDV system remained stable for over 2 years.

After passage 44, the cell-virus interaction entered the third and current (passage 80) phase. The TR virus ceased to be temperature sensitive. In addition, both the CC and TR viruses increased to a similar level of virulence. Although the properties of the late system TR virus are fundamentally identical to the properties of the CC released virus, temperature induction still results in a 2 log increase of released virus. Therefore, it appears that the properties of the original TR virus have changed while the virus was in a repressed state.

Either concomitant or coincidental with the loss of the temperature sensitivity and an increase in virulence of the TR virus was the apparent breakdown of latency as exemplified by the release of virus at 39 C. The establishment and maintenance of the latent infection appears to involve a complex relationship between cell-specific and virus-specific factors. HEF cell-specific factors include an inherent nonpermissiveness to measles virus, the proliferative activity of the cells, and the selection for those cells that can survive infection. Virus-specific factors may include a selection for temperature-sensitive viruses which may interfere with replication of the more virulent particles (CC) isolated from this system.

The observation that measles virus produces a more efficient infection in proliferating cells has potential implications for understanding the neuropathology of measles infections. Measles virus may be maintained in a latent form in the nonproliferating cells of the nervous system. However, the latent virus may be acti-

vated after an event that stimulates proliferation of the cells. This hypothesis is also supported by the observation of other investigators that cells isolated from SSPE patients spontaneously released measles virus after extended in vitro propagation of the brain cells (7). Gliosis is present in SSPE (13) and is characteristic of many demyelinating diseases (6). Whether proliferation of the glia represents an activator of the demyelinating disease or is merely a result of the progression of the disease is not known at present. It is possible that the type of virus activation observed in our latent system may be a natural event in human neuropathology that we can observe on an accelerated time scale due to the frequent proliferation of the latently infected cells maintained in cell culture.

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