The Role of Defective Interfering Particles in Persistent Infection of Vero Cells by Measles Virus

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SUMMARY

Persistent infections by measles virus were rapidly established in the majority of Vero cells when monolayers were infected with virus stocks that had been passed three to five times from an undiluted inoculum. These virus stocks had low infectivity titres but normal haemagglutinin titres and were able to cause interference. The ability of such virus stocks to establish persistent infections seems to be due to the presence of defective interfering particles rather than of virus mutants. Measles virus released from a persistently infected Vero cell line at the 93rd passage had properties similar to the undiluted passage virus that generated persistent infections.

INTRODUCTION

The possible involvement of measles virus in neurological diseases such as subacute sclerosing panencephalitis and multiple sclerosis has generated interest in studies on persistent infection of mammalian cell cultures by measles virus (Rima & Martin, 1976).

It is well established that passage from undiluted inoculum of influenza virus (Von Magnus, 1954) or measles virus (Norrby, Chiarini & Marusyk, 1970) results in changes in the biological characteristics, including the appearance of autointerfering activity, and in the time of development and type of c.p.e. Also, short nucleocapsids with small RNA strands are found in cells infected with undiluted passage virus stocks of measles virus (Kiley, Gray & Payne, 1974). Hall, Martin & Gould (1974) reported accumulation of defective interfering (DI) particles of measles virus.

It has been suggested that DI particles play a role in the establishment of persistent infections (Huang & Baltimore, 1970). On the other hand, Preble & Youngner (1975) suggest that temperature-sensitive (ts) mutants play a role in this process.

The purpose of this paper is to establish whether the rapid onset of persistent infection that we observed in Vero cells infected with undiluted passage stocks of measles virus, was due to the accumulation of mutants or of DI particles. The following experiments indicate that these persistent infections are likely to result from the interaction of measles virus with DI particles.

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METHODS

Viruses. We obtained the Edmonston B strain of measles virus adapted to growth in Vero cells from Dr E. Gould, Department of Microbiology, Queen's University of Belfast, and we term this MV-I. Since Gould (1974) has shown that this strain of measles virus is heterogeneous with regard to plaque morphology, we tried to obtain homogeneous pools of virus by both passage at limiting dilution and by plaque purification. A pool of virus was prepared by Dr P. V. Shirodaria, Department of Microbiology, Queen's University of Belfast, after 9 successive passages at limiting dilution and this we term MV-P9. Subsequently we prepared from MV-P9, by two successive plaque purifications, a further pool of virus which we call MV-A3. These three virus stocks are examples of 'standard virus', as proposed by von Magnus (1954), as they have been passed from diluted inoculum.

Cells. Vero cells were obtained from the Flow Laboratories, Glasgow, Scotland, and were routinely subcultured in Eagle's medium (BHK) supplemented with 5% heat-inactivated calf serum and streptomycin, penicillin and neomycin at 50 units/ml each.

Virus assays. Plaque assays were carried out in monolayers of Vero cells in 40 cm² bottles using a liquid overlay of Eagle's medium supplemented with 2% calf serum. Plaques were counted 3 to 4 days post infection after staining of the monolayers with a saturated solution of methylene blue in 10% formaldehyde. Haemagglutination tests were performed as described earlier by Hall & Martin (1973), except that virus dilutions were made in phosphate buffered saline (PBS) supplemented with 1.6 M-ammonium sulphate for determination of salt-dependent haemagglutination (SDA; Schluederberg & Nakamura, 1967).

Conditions of infection. All experiments described involve monolayers of approx. 5 × 10⁶ Vero cells grown in 40 cm² medical flat bottles. Routinely, one confluent monolayer of Vero cells in a Roux bottle was split into 12 to 15 forty cm² bottles. On the day after subculture, monolayers were washed with pre-warmed PBS and 1.0 ml of virus solution was added. Virus was adsorbed at 37 °C. During adsorption the bottles were rocked mechanically. After 2 h, the inoculum was poured off and 12.5 ml of pre-warmed medium was added.

Undiluted passage. One ml of a virus stock was used to infect a 40 cm² monolayer of Vero cells. Fresh medium was added daily. After development of c.p.e. medium was harvested at daily intervals until the monolayer disintegrated, or for 3 to 4 days in the later passages when persistent infections occurred. For further passage a fresh monolayer of Vero cells was infected with 1 ml of the pooled harvests of the previous passage.

Materials. Eagle's medium was obtained from Wellcome Research Laboratories, England; calf serum from Gibco Biocult, Scotland; neomycin sulphate from Upjohn Company, England, penicillin and streptomycin sulphate from Glaxo Laboratories, England.

RESULTS

Effect of undiluted passage on heterogeneous and homogeneous virus stocks

During attempts to generate DI particles of measles virus by passage from undiluted inoculum in Vero cells, we observed that after five passages of MV-I, the monolayers did not disintegrate and most cells remained attached to the glass and could be subcultured. These cells were shown to be persistently infected with measles virus by three criteria: (1) resistance to superinfection by measles virus but susceptibility to heterologous viruses; (2) detection of measles virus antigens in the cells by immunofluorescence microscopy, and (3) spontaneous occurrence of typical c.p.e. of measles virus at various periods, especially during the early history of the cell line (here termed Vero/MV-I).
DI particles in persistent measles infections

The rapidity with which persistent infection was established in nearly all the cells in Vero monolayers infected with undiluted passage measles virus suggests that selection of host cell mutants is not involved in this process.

In order to determine whether persistently infected cells can be established with a homogeneous preparation of measles virus, undiluted passage series were carried out with three virus preparations, MV-1, MV-P9 and MV-A3. Fig. 1 shows that with each of these the infectivity titres dropped by 2 to 4 log units after the first few passages and then continued at a level between $10^{2}$ and $10^{4}$ p.f.u./ml. On the other hand the salt-dependent haemagglutinin titres remained approximately at the same level (1:2 to 1:8) in all the virus passages, suggesting that large numbers of non-infectious particles accumulated.

Syncytia were seen in the first passage at about 16 h after infection, but in the later passages not until 40 to 50 h after infection. This delay was accompanied by a change from the formation of large spreading syncytia (Fig. 2a) to a smaller strand-forming type of c.p.e. (Fig. 2b), similar to that described by Seligman & Rapp (1959) and Oddo, Flaccomio & Sinatra (1961).

With all three virus stocks, we observed a change from lytic to persistent infection after several undiluted passages. For example, during the fourth passage of MV-A3, 60 to 80% of the cells remained attached to the glass and were confluent after 7 days. In contrast, in the third passage, there was nearly complete disintegration of the monolayer; less than 5% of
the cells remained attached to the glass after 7 days and these cells did not grow to confluence nor could they be subcultured. The change from lytic to persistent infection occurred at similar passage levels with the three virus stocks and was therefore independent of the homogeneity of the strain.

**Effect of diluted passage and plaque purification on undiluted passage virus stock**

The sixth undiluted passage of MV-A3 (MV-A3-UP₆) was passed twice at limiting dilution (LDP₁, LDP₂) and also plaque purified (PPF). The ability of these virus stocks to establish persistent infection was assayed by estimating the percentage of cells that survived the initial onset of c.p.e. Table 1 shows that the virus passed twice at limiting dilution (LDP₂) and also the plaque purified virus had lost the ability to establish persistent infections. The LDP₁ stock obtained by passage of MV-A3-UP₆ at 1:10⁶ dilution gave rise to persistent infection when applied undiluted but when it was diluted to 1:10⁸ the monolayer disintegrated. Thus, the factors in MV-A3-UP₆ that gave rise to the establishment of persistent infections were not diluted out by passage at 1:10⁶ dilution. In fact, as the following experiment indicates, they can accumulate during passage at this dilution. The plaque purified MV-A3 stock (titre 2 × 10⁵ p.f.u./ml) was passed twice from 1:10⁶ diluted inoculum. In 7 out of 9 bottles the virus stripped the cells from the glass within 7 days, and the titre of the released virus was 5 × 10⁴ p.f.u./ml (SDA 1:4). However, in the other 2 bottles, the majority of the cells remained attached to the glass; persistent infections were established and the virus released had a titre of 6 × 10⁵ p.f.u./ml (SDA 1:2).

**Demonstration of interfering components in undiluted passage stocks**

In one series of undiluted passages, we investigated (1) the yield of infectious virus at each passage, (2) the ability to establish persistent infection, and (3) the ability to reduce virus yield in mixed infection with standard virus. Table 2 shows that the infectivity titre dropped rapidly after the third undiluted passage and there was coincidental appearance of persistent infection and a reduction in the yield of virus in mixed infections. With the fifth undiluted passage virus, the reduction in the yield in mixed infection was related to the dilution of the inoculum (Table 2).
Table 1. Effect of limiting dilution passage and plaque purification on the ability of undiluted passage virus (MV-A3-UP6) to establish persistent infection

<table>
<thead>
<tr>
<th>Virus stock</th>
<th>Titre (p.f.u./ml)</th>
<th>Dilution of inoculum</th>
<th>c.p.e. first seen at 48 h</th>
<th>Cells surviving at 11 days (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV-A3-UP6</td>
<td>7.5 × 10^3</td>
<td>1:1</td>
<td>48 h</td>
<td>0-80</td>
</tr>
<tr>
<td>PPF*</td>
<td>6.7 × 10^4</td>
<td>1:1</td>
<td>&lt; 24 h</td>
<td>None†</td>
</tr>
<tr>
<td>LDP1‡</td>
<td>1 × 10^4</td>
<td>1:1</td>
<td>&lt; 24 h</td>
<td>80</td>
</tr>
<tr>
<td>LDP3§</td>
<td>4 × 10^5</td>
<td>1:1</td>
<td>24 h</td>
<td>None</td>
</tr>
</tbody>
</table>

* Obtained by passage of MV-A3-UP6 at 1:10^6 dilution.
† A few surviving cells (< 20 in the monolayer) which grew up to form colonies.
‡ Obtained by plaque purification of MV-A3-UP6.
§ Obtained by passage of LDP1 at 1:10^6 dilution.

Table 2. Interference with undiluted passage virus stocks, assayed by yield reduction tests

<table>
<thead>
<tr>
<th>Virus stock</th>
<th>Titre (log p.f.u./ml)</th>
<th>Persistent infection established</th>
<th>Interference: yield reduction* (log p.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilute passage</td>
<td>6.4</td>
<td>no</td>
<td>-1.0†</td>
</tr>
<tr>
<td>UP3</td>
<td>4.7</td>
<td>no</td>
<td>-0.2</td>
</tr>
<tr>
<td>UP2</td>
<td>6.9</td>
<td>yes</td>
<td>3.1</td>
</tr>
<tr>
<td>UP1</td>
<td>0.7</td>
<td>yes</td>
<td>1.0</td>
</tr>
<tr>
<td>UP5</td>
<td>3.1</td>
<td>yes</td>
<td>1.1</td>
</tr>
<tr>
<td>UP6</td>
<td>3.0</td>
<td>yes</td>
<td>1.8</td>
</tr>
<tr>
<td>UP5 1:10 dilution</td>
<td>---</td>
<td>N.D.‡</td>
<td>N.D.†</td>
</tr>
<tr>
<td>UP3 1:10^8 dilution</td>
<td>---</td>
<td>N.D.†</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* 1.0 ml of each stock was mixed with 1.0 ml of a 1:10^3 dilution of a standard virus stock. Vero cells were inoculated and the virus yield was determined 2 to 5 days p.i. The standard virus alone yielded 4.6 log p.f.u./ml.
† The increased yield (negative reduction) is caused by the large dose of dilute passage virus (1.0 ml) given here.
‡ Not determined.

The interfering activity of undiluted passage virus was also demonstrated by plaque reduction tests. For example, the thirteenth undiluted passage of MV-A3 produced no c.p.e. when applied undiluted to monolayers of Vero cells, at 1:10 dilution it gave rise to small and unclear plaques, and at 1:10^8 dilution produced normal large clear plaques.

The MV-A3-UP13 virus was able to interfere with plaque formation by standard virus and also suppressed the growth of high doses of standard virus that otherwise destroyed monolayers in less than 40 h (Table 3). Monolayers infected with 4 × 10^4 p.f.u./ml standard virus and undiluted MV-A3-UP13 could be subcultured 7 days after infection and were shown to be persistently infected by the criteria mentioned earlier.

It was found that the interfering activity present in MV-A3-UP13 was readily destroyed by ultraviolet radiation and was sedimented by centrifuging at 80000 g for 1 h.
Table 3. Effect of the 13th undiluted passage virus on plaque formation by standard virus

<table>
<thead>
<tr>
<th>Virus applied to monolayers</th>
<th>UP virus dilution*</th>
<th>Plaques formed</th>
<th>Diam. (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard virus (p.f.u.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$4 \times 10^1$</td>
<td>—</td>
<td>39-41</td>
<td>1.8-2.0</td>
</tr>
<tr>
<td>—</td>
<td>Undiluted</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td>$4 \times 10^1$</td>
<td>1:10</td>
<td>~ 200</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>$4 \times 10^2$</td>
<td>1:10²</td>
<td>47-50</td>
<td>1.8-2.0</td>
</tr>
<tr>
<td>—</td>
<td>Undiluted</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td>$4 \times 10^3$</td>
<td>Undiluted</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td>$4 \times 10^4$</td>
<td>Undiluted</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td>$4 \times 10^5$</td>
<td>Undiluted</td>
<td>None</td>
<td>—</td>
</tr>
</tbody>
</table>

* Titre $8 \times 10^2$ p.f.u./ml; SDA 1:8 (1.0 ml applied)
† Monolayer stained lightly.
‡ About 60% of the cells remained attached.

Effect of temperature on undiluted passage measles virus stock

Accumulation of ts mutants in undiluted passage could explain the decrease in infectivity titre (assayed at 37 °C) and the appearance of persistently infected cells. Therefore, we compared the efficiency of plaque formation (e.o.p.) between undiluted passage virus and standard virus at different temperatures. For standard virus the e.o.p. at 33 °C was 71% of the e.o.p. at 37 °C, while for undiluted passage virus, the e.o.p. at 33 °C was only 16% of that at 37 °C (mean result from 4 experiments, with 3 different undiluted passage virus stocks). The reason for the low e.o.p. at 33 °C for undiluted passage virus is not clear, but it was evident that these stocks did not contain a large number of ts mutants with 37 °C as a non-permissive temperature. Fig. 3 shows that there was no appreciable difference between the standard virus and the undiluted passage virus in the e.o.p. at temperatures above 37 °C.

Release of virus from persistently infected cells by temperature shift

The persistently infected cell line (Vero/MV-1) established as described earlier has been maintained in culture for 106 passages over a period of 13 months. At this stage 78% of the cells contained virus antigen as judged by immunofluorescence microscopy. Incubation of this cell line at temperatures below 37 °C did not lead to an increase in the titre of released virus. However, when these cells were incubated at 40 °C for 24 h and subsequently at 37 °C, the culture entered a lytic crisis, typical measles c.p.e. spread throughout the monolayer and the titre of the released virus increased approx. 40 to 50-fold. Such lytic crises were induced in five separate experiments but the time between temperature shift down and disintegration of the cell sheet varied from 3 to 12 days. We termed the virus released by temperature shift from the 93rd passage of Vero/MV-1 cells, MV-1px (infectivity titre $4.4 \times 10^2$ p.f.u./ml). This virus stock was passed twice at 1:10 dilution in order to increase the titre of the stock. Resulting virus stocks contained large amounts of DI particles, as c.p.e. was absent when monolayers were infected with undiluted inoculum and very little c.p.e. occurred at 1:10 dilution. The presence of DI particles was also evident from the fact that this virus stock
interfered with plaque formation by standard virus. The interfering activity was again sensitive to u.v. irradiation. Passage of this virus stock at 1:10^4 dilution removed the interfering activity and the ability to establish persistent infection.

**DISCUSSION**

We have demonstrated that measles virus, after serial undiluted passage, can readily establish persistent infections in a majority of the cells in Vero monolayers. This phenomenon has been noted earlier by Norrby *et al.* (1970) and is here described in detail. The rapid manner in which a large majority of the cells become persistently infected makes it unlikely that the selection of resistant host cells plays a role in this process. Also, as Vero cells do not synthesize interferon (Desmyter, Melnick & Rawls, 1968), it is unlikely that this plays a role here.

The ability of measles virus to establish persistent infections appears to be related to the nature of the virus inoculum. From the low ratio of the infectivity to haemagglutination titres of UP virus stocks, it can be concluded that they contain large numbers of non-infectious particles and these are able to interfere with the development of standard measles virus (Tables 2 and 3). The interfering activity appears to reside in a large, presumably RNA-containing particle as it is sensitive to u.v.-light and sedimented by ultracentrifugation. Hall *et al.* (1974) have shown that DI particles accumulate after several undiluted passages of measles virus, and the results presented here support these earlier observations and link the appearance of DI particles to the ability to establish persistent infection in our Vero/measles system (Table 2).
When UP virus stocks are passed under conditions that may lead to a reduction in the numbers of DI particles such as passages with 1:10⁴ diluted inoculum or after plaque purification, both interfering activity and the ability to establish persistent infections are lost (Table I). It therefore appears that DI particles play a role in the establishment of persistent infection of Vero cells by measles virus. Our results are in agreement with those obtained for persistent infection of BHK cells by vesicular stomatitis virus (Holland & Villarreal, 1974) and by rabies virus (Kawai, Matsumoto & Tanabe, 1975), and for persistent infection of Vero cells by canine distemper virus (ter Meulen & Martin, 1976).

We were unable to demonstrate accumulation of large numbers of ts mutants in our UP stocks. Furthermore, persistent infections were established as readily after a few undiluted passages of homogenous stocks (MV-A3 or MV-P9) as with the parent stock MV-1. These observations indicate that mutants probably do not play a role in the establishment of persistent infections in our Vero/measles system. We have recently reviewed the involvement of mutants or DI particles in a number of persistent infections of tissue culture cells by RNA viruses (Rima & Martin, 1976).

Virus released from persistently infected cells may not necessarily be representative of the intracellular virus (Rima & Martin, 1976). Nevertheless, the results presented here indicate that virus stocks that are able to establish persistent infections have similar properties to those released from persistently infected cells after many subcultures. The released virus appears to contain DI particles, and its ability to establish persistent infection is lost after passage with diluted inoculum or after plaque purification.

In conclusion, we suggest that a number of well documented variations in characteristics of measles virus such as plaque morphology and type of c.p.e. (Seligmann & Rapp, 1959; Oddo et al. 1961), cytolytic ability of the virus, localization of intracellular immunofluorescence and virus yield (Hamilton, Barbosa & Dubois, 1973), ability to establish persistent infection and buoyant density of virus on gradients (Norrby et al. 1970; B. K. Rima & S. J. Martin, unpublished results) may be explained by interaction of standard virus with DI particles. Our observations indicate that DI particles can be maintained at high concentration or accumulate during passage at 1:10⁴ diluted inoculum. Also, during plaque purification the opportunity arises for multiple cycles of infection during which DI particles can accumulate. Hence, observations on plaque morphology could be explained by the presence of DI particles especially when the plaque characteristics are not maintained on further passage. Similarly, during passage at limiting dilution, multiple cycles of virus growth take place which again can lead to accumulation of DI particles towards the end of the growth period. The best procedure we have found for obtaining measles virus preparations which are minimally contaminated with DI particles is passage at an intermediate dilution of 1:10³ so that the growth period is relatively short.

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REFERENCES


DI particles in persistent measles infections


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