Immediate Persistent Infection by Human Parainfluenza Virus 3: Unique Fusion Properties of the Persistently Infected Cells

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SUMMARY

We describe here a persistently infected cell system with unique properties. Cells infected with human parainfluenza virus type 3 (PF3) at high multiplicities of infection showed little or no cytopathic effects (cell fusion). Unlike other paramyxovirus persistent infections that require a long development time, the majority of the cells survived the initial infection and formed persistently infected cell cultures that were immediately available for study. In addition, unlike other paramyxovirus persistent infections, the PF3 system described here produced high levels of infectious virus and did not undergo periodic crises. Although cells persistently infected with PF3 contained large amounts of the cleaved, active form of the viral fusion protein, F₁, the persistently infected cells did not fuse with each other. However, they did fuse with uninfected cells within minutes of cell-to-cell contact. Other persistent paramyxovirus infections do not have this property. Fusion occurred with all cells tested, including red blood cells, and was not dependent on protein synthesis. The unique fusion properties of these PF3 persistently infected cells make this an interesting system for the study of mechanisms of viral fusion and mechanisms of inhibition of viral fusion.

INTRODUCTION

Human parainfluenza virus type 3 (PF3), a member of the family Paramyxoviridae, is the aetiological agent of a severe lower respiratory illness in infants (Tyeryar, 1983; Welliver et al., 1982). Despite its importance as a human pathogen, analysis of PF3 has only recently been carried out. There have been reports on intracellular PF3 protein synthesis (Wechsler et al., 1985a), virion proteins and structure (Storey et al., 1984; Wechsler et al., 1985b, c; Sanchez & Banerjee, 1985), and cloning and sequencing of viral genes (Galinsky et al., 1986a, b, c; Elango et al., 1986; Spriggs et al., 1986; Sanchez et al., 1986).

Persistent infection with paramyxoviruses has become an area of increasing interest because of the involvement or suspected involvement of these viruses in several chronic diseases of man. Persistent measles virus infection causes the slow neurological disease subacute sclerosing panencephalitis (see Wechsler & Meissner, 1981; Morgan & Rapp, 1977 for reviews). Persistent measles infection has also been implicated in multiple sclerosis (Morgan & Rapp, 1977). Persistent infections with respiratory syncytial, measles, simian virus 5 and PF3 viruses have been implicated in Paget’s disease of bone (Goswami et al., 1984; Mills et al., 1981). The mechanism(s) by which paramyxoviruses can persist in cells has not been well elucidated, and much work remains to be done in this area.

Although there is no evidence that PF3 persistence causes disease in humans, there have been reports indicating that PF3 causes persistent infections in man (Frank et al., 1981; Muchmore et al., 1981; Gross et al., 1973; Goswami et al., 1984). In this report, in addition to demonstrating
some unique fusion properties of cells persistently infected with PF3, we also show the ease and reproducibility with which PF3 virus can establish and maintain stable persistent infections in cell culture. This system is attractive because unlike other paramyxovirus persistent infections that require a long development time, under the proper conditions PF3 persistence is established immediately. This will allow examination of the initial events that occur in the establishment of persistence. In addition, unlike other paramyxovirus persistent infections (Rustigian, 1966a, b; Rima & Martin, 1976; Wechsler & Meissner, 1981) the PF3 system described here produces very high levels of infectious virus \((5 \times 10^6\) to \(10^8\) p.f.u./ml) and does not undergo periodic crises. These factors combine to make PF3 a unique and interesting model of viral persistence.

**METHODS**

**Cell lines and virus.** CV-1 cells, Vero cells, HeLa cells, and HEp-2 cells were grown as monolayers as previously described (Wechsler & Fields, 1978). Virus stocks were also prepared as previously described (Wechsler et al., 1985a). Briefly, human parainfluenza virus type 3 (PF3; HA-l, NIH No. 47885, cat. no. V323-002-020) was plaque-purified three successive times, and stocks were prepared by infecting CV-1 monolayers with a freshly picked plaque. At 36 to 48 h post-infection, virus was harvested by freezing and thawing.

**Indirect viral immunofluorescence.** This was carried out using hyperimmune rabbit sera and fluorescein-conjugated anti-rabbit IgG as previously described (Wechsler et al., 1979).

**Infectious centre assays.** These were carried out using CV-1 cells as indicator cells as previously described (Wechsler et al., 1979).

**Testing for interferon.** Culture fluids were acid-treated (pH 2.0, overnight, 4 °C), neutralized and applied for 4 h to cell monolayers which were then used in vesicular stomatitis virus and PF3 plaque reduction assays.

**Analysis of RNA to detect subgenomic (defective interfering, DI) particles.** Labelled and unlabelled virion, nucleocapsid and intracellular viral RNAs were purified and their size was determined on gels (by autoradiography or staining) as previously described (Wechsler et al., 1985a). Sizing of virion and intracellular viral RNAs was also done by Northern blot analysis using nick-translated PF3 cDNA clones as probes as previously described (Galinsky et al., 1986a).

**Isotopic labelling of cells, purification of virions, preparation of cell extracts and SDS–PAGE.** These were as previously described (Wechsler et al., 1985a, b, c).

**RESULTS**

**Establishment and characterization of persistent infections**

Infection of CV-1 cells with PF3 at low m.o.i. caused lytic infections with extensive c.p.e. and cell destruction. By 24 h after infection at an m.o.i. of 0.01 to 1 (Fig. 1, day 1), approximately 50% of the cells were involved in cell fusion (c.p.e.). By day 5 only a few scattered cells remained. Fig. 1, day 5, m.o.i. 0.01 to 1, shows those areas of the monolayers containing the most surviving cells. In contrast, at an m.o.i. of 10, no c.p.e. was seen 24 h after infection (Fig. 1, day 1). At 5 days after infection at an m.o.i. of 10, the majority of the cells remained viable and unfused. Fig. 1, day 5, m.o.i. 10 shows the part of the monolayer containing the fewest surviving cells. Although the cells shown here on day 5 appeared unhealthy, they grew at the same rate as uninfected cells, and within a few days were visually indistinguishable from uninfected cells.

In experiments with CV-1, HEp-2, HeLa and Vero cells, at least 50% and usually over 90% of the cells remained free of c.p.e. following infection at high multiplicity with PF3 virus. These cells were persistently infected by the following criteria: (i) indirect immunofluorescence using hyperimmune rabbit sera indicated that over 95% of the cells contained PF3 antigens (not shown); (ii) c.p.e. developed when monolayers were challenged with wild type (wt) PF3 virus, although these cells continued to support the growth of other paramyxoviruses (measles virus and respiratory syncytial virus) at normal levels and with normal c.p.e. and cell destruction (not shown); (iii) infectious centre assays indicated that over 90% of the cells contained virus capable of making plaques on fresh cells (not shown); (iv) persistent cultures could be grown from five of five individually cloned cells; (v) the persistently infected cells shed virus \((5 \times 10^6\) to \(1 \times 10^8\) p.f.u./ml) into the medium.

Thus, following infection at high multiplicity, PF3 virus rapidly produced persistent infections in which most of the cells survived the initial infection and were therefore immediately available for study. This differs from the usual manner of establishing persistence...
Fig 1. Establishment of immediate persistent infection of PF3 virus in CV-1 cells. CV-1 cell monolayers were infected with four times plaque-purified PF3 virus at an m.o.i. of 0.01, 0.1, 1 or 10 p.f.u./cell as indicated at the top of the figure. At 24 h post-infection (day 1) and 5 days post-infection (day 5) photographs of the cells were taken through a phase contrast microscope. The photographs in the top row (day 1) show representative fields. The first three photographs in the bottom row (day 5, m.o.i. 0.01, 0.1, 1) are selected fields showing areas containing the largest numbers of surviving cells. In contrast, the last photograph (day 5, m.o.i., 10) is a selected field showing an area containing the fewest surviving cells.

in which the initial infection kills most of the cells and the few survivors are grown into a culture over a prolonged period of time. We have given this phenomenon, in which there is no time lag between establishment of persistence and the ability to study the culture, the descriptive name 'immediate persistent infection'.

The ability to generate immediate persistent infections, combined with two other unique properties, make this system an excellent model for the study of paramyxovirus persistence. (i) Unlike most other persistent systems that exhibit periodic crises of c.p.e. and cell destruction, the PF3 persistently infected cultures were stable. In over 2 years of continued growth (over 100 passages) of several PF3 persistent cultures, crises did not occur. (ii) The PF3 persistently infected cells produced large amounts of virus and viral products, compared to other persistent systems that have been described. In five persistently infected cultures we have examined, PF3 yields ranged from $5 \times 10^6$ to $10^8$ p.f.u./ml. This is 1000-fold higher than other paramyxovirus persistent infections (Rima & Martin, 1976; Wechsler & Meissner, 1981).

**Lack of DI particles**

The establishment of persistent infection only at high m.o.i. suggested the possibility that DI particles might be involved. In addition, immediate establishment of persistence was previously observed upon infection of BHK and LLC-MK2 cells with a mixture of standard and DI particles of Sendai virus (Roux & Holland, 1979). We therefore prepared and tested viral stocks under conditions that minimized the possibility of generation or amplification of DI particles. Starting with our standard three times plaque-purified virus stock, PF3 was plaque-purified, individual picked plaques were suspended in buffer, replated and individual plaques were picked again. This procedure was repeated until four successive cycles of plaque purification were carried out without intervening viral passages that might allow generation of DI particles. Stocks were grown from several of these isolates by a single passage in CV-1 cell monolayers at m.o.i. of less than 0.01. Subsequent infection of cells at a high multiplicity with these viral stocks resulted in immediate persistent infections as described above.

DI particles could not be detected in these or other less stringently produced viral stocks, or in persistently infected CV-1 cells (PF3/CV-1) or persistently infected HEp-2 cells (PF3/HEp-2),
Fig. 2. Kinetics of fusion of CV-1 cells added to a monolayer of PF3/CV-1 cells. A confluent monolayer of CV-1 cells from one 25 cm² flask was trypsinized and the total cell suspension was added to a confluent monolayer of PF3/CV-1 persistently infected cells in a second 25 cm² flask. At various times after addition of the CV-1 cells photographs of the cells were taken through a phase contrast microscope. Approximately 30 to 45 min was required for the added cells to settle through the medium and come into contact with the PF3/CV-1 monolayer. (a) PF3/CV-1 cells prior to the addition of CV-1 cells; (b) 15 min after addition of CV-1 cells; (c) 30 min; (d) 45 min; (e) 60 min; (f) 90 min.

even after 2 years in culture. Labelled or stained virion RNA, nucleocapsid RNA and total cellular RNA from long term persistently infected cultures and from cells acutely infected at high m.o.i. were analysed for size on gels as described in Methods. No subgenomic RNAs (other than mRNAs in cell extracts) could be seen (data not shown). Subgenomic RNAs also could not be found by probing Northern blots with PF3 cDNA clones (not shown). Thus, DI's do not appear to play a major role in these persistent infections.

Lack of temperature-sensitive (ts) mutants and interferon

Other factors often thought to be involved in persistent infections include interferon and ts mutants. Fifty plaques from PF3/CV-1 persistently infected cells were tested and none were ts. Interferon could not be detected in either PF3/CV-1 or PF3/HEp-2 cells. In addition, immediate persistent infections could be established in Vero cells, which cannot make interferon. Thus, it is unlikely that either ts mutants or interferon plays a significant part in the mechanism of these persistent infections.

Rapid fusion of persistently infected cells with uninfected cells

One intriguing characteristic of the PF3 persistent infections is that even though they do not show any cell fusion themselves, they are able to fuse rapidly with normal cells. Other paramyxovirus persistent infections do not have this property. Uninfected CV-1 cells were trypsinized and added to a monolayer consisting of an equal number of PF3/CV-1 cells. Fig. 2(a) shows the PF3/CV-3 cells just prior to the addition of CV-1 cells. Fig. 2(b to f) shows the formation of cell fusion at 15, 30, 45, 60 and 90 min after the addition of uninfected cells. Cell fusion occurs within minutes of the initial contact between the uninfected and the persistently infected cells. (It takes about 30 to 45 min for the cells to settle through the medium.) By 60 to 90 min, virtually all the cells in the monolayer are involved in fusion (e and f). Identical results were...
Fig. 3. Fusion of PF3/CV-1 cells with different cells and in the presence of various inhibitors. Uninfected cells were added to PF3/CV-1 cell monolayers as described in the legend to Fig. 2. All photographs were taken 2 h after the addition of cells. (a) Added HEp-2 cells; (b) added HeLa cells; (c) added CV-1 cells that had been suspended with EDTA without trypsin; (d) PF3/CV-1 cells were pretreated with 1 μg/ml cycloheximide for 15 min and fused with CV-1 cells in the same concentration of cycloheximide. (e) PF3/CV-1 cells were pretreated with 1 μg/ml CCCP for 15 min and fusion with CV-1 cells was carried out in the same concentration of CCCP. (f) The PF3/CV-1 monolayer was subjected to 0.03 J of u.v. prior to addition of CV-1 cells. This dosage was sufficient to kill similarly treated cell monolayers as determined by visual examination (the cells rounded up and came off the substrate within 4 to 6 h).

obtained in the complementary experiment in which PF3/CV-1 cells were added to uninfected CV-1 cells (data not shown).

Identical fusion occurred with all cells tested. HEp-2 cells (Fig. 3a), HeLa cells (Fig. 3b), Vero cells, LLC-MK2 cells, and even red blood cells (data not shown) all fused when added to PF3/CV-1 cells. The fusion protein of paramyxoviruses is synthesized in an inactive form (F0) that is activated by cleavage with a trypsin-like enzyme. Thus, it was possible that the fusion was due to cleavage of F0 by the trypsin used to detach cells from flasks prior to mixing the uninfected and persistently infected cells together. Since we routinely passaged the PF3/CV-1 and PF3/HEp-2 cultures using trypsin and have never detected any fusion in these cultures we thought that this was unlikely. However, to rule out any effects of trypsin rigorously, CV-1 cells were brought into suspension using EDTA without trypsin. Addition of these cells to a PF3/CV-1 cell monolayer gave identical fusion (Fig. 3c).

To study the possible role of new synthesis of macromolecules, fusion was attempted in the presence of various inhibitors following pretreatment of the PF3/CV-1 cells. Neither cycloheximide, which blocks protein synthesis, nor carbonyl cyanide m-chlorophenylhydrazone (CCCP), which blocks oxidative phosphorylation, affected the rate or the extent of fusion (Fig. 3d and e). Long term treatment with actinomycin D, which blocks cellular DNA synthesis also had no effect (data not shown). In addition, subjecting PF3/CV-1 cells (Fig. 3f), uninfected cells, or both (data not shown) to massive, lethal amounts of u.v. had no effect on fusion. It therefore appears that the fusion is a membrane phenomenon that requires no additional energy or new macromolecule synthesis. These results also indicate that even though the persistently
infected cells cannot fuse with each other, large amounts of functional fusion protein are present in these cells.

**Fusion with other persistently infected cells**

Fig. 4 shows experiments in which different persistently infected cells were added to PF3/CV-1 persistently infected cells. When a second PF3/CV-1 cell line was added no fusion occurred (Fig. 4a). Similarly, when PF3/HEp-2 persistently infected cells were added no fusion occurred (Fig. 4b). This suggests that the mechanism blocking fusion in all of these PF3 persistent infections is the same. In contrast, adding a cell line persistently infected with measles virus (Fig. 4c) or a cell line persistently infected with respiratory syncytial virus (Fig. 4c) to PF3/CV-1 cells resulted in fusion identical to that seen with the addition of uninfected cells. This indicates that cells persistently infected with measles virus or respiratory syncytial virus do not block fusion by a mechanism similar to that exhibited by PF3 persistent infections.

**Resistance to fusion of newly established persistent infections**

We determined at what point newly established persistent infections became resistant to fusion by added PF3/CV-1 cells. CV-1 cells were infected with PF3 at an m.o.i. of 10. At intervals, the monolayers were challenged with PF3/CV-1 cells. Fusion was monitored 60 min later. Uninfected cells and cells at time 0 after infection (following a 2 h adsorption period) were completely fused by added PF3/CV-1 cells (Fig. 5a, b). However, as early as 1 h after infection, a
PF3 persistence: unique fusion properties

Fig. 5. Ability of newly established PF3/CV-1 persistently infected cells to resist fusion with established PF3/CV-1 cells. Flasks of CV-1 cells were infected with PF3 at an m.o.i. of 10. At various times post-infection, PF3/CV-1 cell suspensions were added to the flasks as described in the legend to Fig. 3. Photographs were taken 60 min after addition of cells. (a) uninfected CV-1 cells; (b) 0 min post-infection; (c) 1 h; (d) 3 h; (e) 5 h; (f) 7 h; (g) 9 h; (h) 12 h; (i) 16 h post-infection.

few cells were resistant to being fused (Fig. 5c). By 5 h after infection the newly infected cells acted as in a long term persistent infection and were completely resistant to fusion by added PF3/CV-1 cells (Fig. 5f). Thus, it appeared that partial resistance to fusion occurred with little or no viral protein synthesis, but that viral protein synthesis was required for complete protection. This was confirmed by infecting cells with u.v.-inactivated virus which could not synthesize viral proteins. Similar to the resistance seen at 1 h after infection with live virus, only 1 to 5% of cells infected with u.v.-inactivated virus became resistant to fusion, regardless of the time after infection (not shown).

Fusion by newly established persistent infections

We also examined the ability of newly established persistent infections to fuse with added uninfected cells. CV-1 cells were infected with PF3 virus at an m.o.i. of 10. As described above, no fusion was seen at any time within the first 24 h after infection (see Fig. 1, day 1, m.o.i., 10). At intervals, the cells were challenged with uninfected CV-1 cells and fusion was monitored 60 min later (data not shown). Uninfected cells and cells within the first 5 h post-infection did not fuse. However, as early as 7 to 9 h after infection, the addition of uninfected cells resulted in detectable fusion. By 12 to 16 h, addition of uninfected cells resulted in a high level of fusion.
The 7 to 9 h time period corresponds to the time after infection at which viral protein synthesis can be readily detected (Wechsler et al., 1985a). In addition, we found that if new viral protein synthesis was blocked with cycloheximide, or if cells were infected with u.v.-inactivated virus, added uninfected cells were not fused (not shown). In contrast, pretreatment of cells prior to and during infection with actinomycin D did not affect their ability to fuse added uninfected cells. Thus the ability of these cells to fuse added uninfected cells appears to be dependent on synthesis of viral, but not new cellular, proteins.

Thus, shortly after infection under conditions that lead to the rapid establishment of persistence in most of the cells in the culture, the cells were able to fuse with normal cells and were resistant to fusion with PF3/CV-1 cells. Therefore, within hours the cells were functioning in a manner equivalent to long term persistently infected cells. This lends further support to the notion that persistence is established 'immediately'.

**Viral proteins**

$[^{35}S]$Methionine labelling of intracellular proteins followed by SDS–PAGE was used to examine total viral protein synthesis in cells persistently infected with PF3 (Fig. 6, lanes 2, 3). The persistently infected cells shown here were used between 5 and 7 days after the initial infection at high multiplicity used to establish persistence. Several differences were seen between viral proteins synthesized in acute, lytic infections (lane 1) compared to persistently infected cells (lanes 2 and 3). In one of the two cell lines shown here (lane 2), the viral M (matrix) protein migrated more slowly than the wt M protein. We found this to be the case in two of five cell lines examined. In all five persistently infected cell lines examined, a new, or more intensely labelled band, designated X, appeared just above M (lanes 2 and 3). This band might be an up-regulated host protein, as there is a very faint cellular band in this region (not shown). X might also be a degradation product of another viral protein. However, at this time we do not know the origin of this band.

The fusion glycoprotein was of particular interest to us because of the biological observations of cell fusion reported above. The PF3 glycoproteins are most easily analysed following $[^{3}H]$mannose labelling (Wechsler et al., 1985a, b). Therefore, virion glycoproteins and intracellular viral glycoproteins were examined (in the same two persistently infected cell lines shown in lanes 2 and 3) following $[^{3}H]$mannose labelling. Virions from both persistent infections (lanes 5 and 6) contained very little of the uncleaved non-functional precursor fusion protein F₀ compared to wt virions (lane 4). On the other hand, virions from both persistently infected cell lines contained much more of the cleaved functional fusion protein F₁ than did wt virions. Although it is more difficult to see, the intracellular glycoproteins followed this same pattern (lanes 8 and 9). Thus, the PF3 persistently infected cells contained a lower fraction of uncleaved F₀ and a higher fraction of cleaved F₁ than did lytically infected cells. We found the same fusion pattern in all five persistently infected cell lines examined. This included cells persistently infected for 2 years and cells examined during the first 36 h following the initial infection at high multiplicity (data not shown).

**DISCUSSION**

Paramyxovirus persistent infections in tissue culture have classically been established by infecting cells at high or low m.o.i. with standard virus, of standard virus plus DI virus (Wechsler & Meissner, 1981; Rima & Martin, 1976; Roux & Waldvogel, 1981). An acute lytic infection generally occurs in which most of the cells are destroyed. The few surviving cells must then be carefully nurtured to produce a persistent culture. The time for this regrowth into a culture containing a usable number of cells is measured in months. Several difficulties make these persistent infections refractory to analysis of events involved in the establishment of persistence. (i) Of necessity, a long time elapses between the initiation of persistence and analysis of the persistently infected cells. This makes it difficult, if not impossible, to differentiate between alterations that may have caused the persistent infection and alterations that are the result of the persistent infection. (ii) These persistently infected cultures go through periodic crises in which c.p.e. develops, most of the cells lyse and, similar to what happened
Fig. 6. SDS-PAGE of [35S]methionine-labelled and [3H]mannose-labelled virion and intracellular proteins from cells persistently infected with PF3. Persistently infected cell monolayers were labelled with [35S]methionine or [3H]mannose as previously described for acutely infected cells (Wechsler et al., 1985a, b). Extracellular virions and total cellular extracts were harvested and processed for SDS-PAGE and fluorography as previously described (Wechsler et al., 1985a, c). Persistently infected cultures were used between 5 and 7 days after the initial establishment of the infection. Control acute infections were done at an m.o.i. of 0.1 and analysed between 18 and 36 h post-infection. [35S]Methionine labelling was done for 2 h; [3H]mannose labelling was for 24 h. Gel lanes: (1) [35S]methionine-labelled total cell extract from acutely infected cells; (2) [35S]methionine-labelled total cell extract from a persistently infected cell line; (3) [35S]methionine-labelled total cell extract from a second persistently infected cell line; (4) [3H]mannose-labelled virions from acutely infected cells; (5) [3H]mannose-labelled virions from the persistently infected cell line shown in lane 2; (6) [3H]mannose-labelled virions from the persistently infected cell line shown in lane 3; (7) [3H]mannose-labelled total cell extract from acutely infected cells; (8) [3H]mannose-labelled total cell extract from the persistently infected cell lines shown in lanes 2 and 5; (9) [3H]mannose-labelled total cell extract from the persistently infected cell lines shown in lanes 3 and 6.

during the initial establishment of persistence, repopulation of the culture occurs from the outgrowth of a few surviving cells. This phenomenon may result in the selection of a new persistently infected population following each crisis period (Wechsler & Meissner, 1981). (iii) Often, cultures do not survive a crisis and the persistent infection is lost. In many instances this makes it impossible to study persistent infections over extended periods. (iv) Most persistently infected cells synthesize viral material (genomic RNA, mRNA, proteins, virions) at very low rates. This makes biochemical and even biological analysis difficult.

The PF3 persistent system described in this report has none of these difficulties. Under appropriate conditions, PF3 produces 'immediate' persistent infections in which most of the cells survive the initial infection and are therefore immediately available for study. The
Persistently infected cultures are stable and do not undergo periodic crises. Finally, the PF3 persistently infected cultures produce large amounts of virus, approximately 1000-fold higher than other paramyxovirus persistent infections (Wechsler & Meissner, 1981; Rima & Martin, 1976).

Establishment of immediate PF3 persistent infections occurs only at high m.o.i. This multiplicity effect suggests some kind of interference phenomenon, such as DI particles. However, viral yields remain high, arguing against a major interference with viral synthesis as would normally occur with DI particles. In addition we have been unable to detect subgenomic length RNAs in this system. However, we have not yet been able rigorously to rule out the presence of small amounts of very small DI particles or large DI particles that co-migrate with full length genome.

One possibility for the multiplicity effect is that at high m.o.i. overproduction of a viral product or products protects the cell from viral killing, perhaps by some feedback mechanism. It is also possible that there is some non-viral protective factor in the inoculum that only becomes apparent when large amounts of the inoculum are used.

The most interesting aspect of our PF3 persistently infected cells is their ability to fuse rapidly with uninfected cells. This fusion occurs within minutes of actual cell–cell contact (about 45 min after cells are added, which is the amount of time it takes the cells to settle through the medium) and is not dependent on new protein synthesis. The speed with which the fusion occurs suggests that the persistently infected cells must contain large amounts of functional fusion protein that is poised for fusion as soon as an uninfected cell membrane is brought into proximity. Furthermore, there must be some mechanism that prevents these apparently functional fusion proteins from fusing the persistently infected cells to other persistently infected cells in the cultures.

We currently have two working hypotheses that we are testing as possible mechanisms of this fusion phenomenon. First, there may be an alteration in the lipid composition of the persistently infected cell membrane which renders them resistant to fusion and which can be overcome by a lipid contribution from a normal membrane. The lipid alteration could be a secondary effect due to a cellular response to large amounts of a viral product. Alternatively, the lipid alteration could be a direct result of large amounts of viral protein being inserted into the membrane.

Second, it is possible that there is too much functional fusion protein in the cells. It is likely that there are specific sites on the adjacent cell membrane with which the fusion protein interacts. If we speculate that the fusion protein is anchored into the persistent cell membrane at or near these sites, then these sites might be physically unavailable if there is an overabundance of fusion protein. A similar situation could occur with an excess of HN, the other surface glycoprotein. In either case, the fusion protein would be perfectly normal and ready to function if its active amino terminal site could find a receptor. This would lead to rapid and extensive fusion when persistently infected cells were mixed with equal numbers of uninfected, susceptible cells. This theory would also explain why infections at low multiplicities which produced less fusion protein) caused cell fusion and infections at very high multiplicities (which produced large amounts of fusion protein) did not.

This hypothesis is supported by the results of our protein analysis. All of the persistently infected cell lines examined, including one cell line examined within 36 h of the initial infection at high multiplicity used to establish persistence, contained more $F_1$ than did lytically infected cells. In all the persistently infected cells, the ratio of $F_1$ to $F_0$ was much higher than in lytically infected cells. These results also indicate that the absence of fusion in the persistently infected cultures was not due to a lack of processing of the fusion protein precursor, $F_0$, into the active form, $F_1$.

In two of the five PF3 persistently infected cell lines examined, the M protein had an altered electrophoretic mobility. This is reminiscent of alterations seen in the M protein during measles virus persistent infections (Wechsler et al., 1979; Wechsler & Meissner, 1981) and these alterations are thought to play a role in persistence (Wechsler & Meissner, 1981). Since no definitive evidence has been found for post-translational modification of the measles virus M protein, M protein alterations have been assumed to be mutations. However, alterations in the
electrophoretic mobility of the M protein in at least one PF3 persistently infected cell line was seen within 36 h of the initial infection at high multiplicity. This is not enough time for a viral mutation to have spread through the population and suggests some sort of pre- or post-translational modification of M.

The finding that readily detected changes occurred in some M proteins very rapidly after the establishment of PF3 virus persistence suggests a relationship between these changes and the establishment of persistence. This lends support to the notion that alterations in M may also be involved in measles virus persistence. Whether the alterations in M are involved in the mechanism of establishment of persistence or are a result of the persistent infection remains to be determined.

Because of the ease and reproducibility with which PF3 establishes and maintains stable persistently infected cell cultures, it is likely that following acute respiratory disease, PF3 might also tend to establish persistent infections in children. Live, attenuated PF3 viral vaccines might also tend to establish such infections. This is, of course, an area of major concern in the development of vaccines. It will be important to determine whether natural PF3 virus infection tends to result in persistence and if so, whether this is detrimental, neutral or beneficial. If detrimental it will of course be important to determine whether a potential vaccine is even more likely to cause a detrimental persistent infection. On the other hand, it is possible that long lasting immunity can only be conferred if the immune system is continually stimulated by viral persistence (and that in other ways PF3 persistence is neutral). In this case potential vaccine strains that are more capable of causing persistence that wt virus would be desirable.

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