Evidence that the paramyxovirus simian virus 5 can establish quiescent infections by remaining inactive in cytoplasmic inclusion bodies

R. Fearns, D. F. Young and R. E. Randall*

School of Biological and Medical Sciences, The Irvine Building, North Street, University of St Andrews, Fife KY16 9AL, U.K.

Introduction

It has been documented extensively that paramyxoviruses can establish persistent infections both in tissue culture cells and in vivo. Persistent infections in vivo may have a number of consequences for both virus and host (reviewed in Randall & Russell, 1991). It is possible that many paramyxoviruses can cause persistent infections, in humans and other animals, that remain silent because they do not induce harmful pathology. Indeed, it has been suggested in the case of measles, that such silent infections may contribute to the maintenance of life-long protective immunity to the virus (Burns & Allison, 1975; ter Meulen & Cartner, 1982). However, there may be other consequences of paramyxovirus persistence. For example, there is some epidemiological evidence that certain individuals may secrete respiratory paramyxoviruses for prolonged periods of time. Also a number of chronic diseases have either been shown to be caused by persistent paramyxovirus infections [e.g. subacute sclerosing panencephalitis (SSPE)] or have been linked with such infections (e.g. Paget's bone disease and autoimmune chronic active hepatitis; reviewed in Randall & Russell, 1991).

Studies examining the events leading to the establishment and maintenance of persistent paramyxovirus infections have, in the main, focused on the role of defective interfering particles or mutants (reviewed in...
Re, 1991). However, it is important to bear in mind that there may be distinct types of persistent paramyxovirus infection. Persistence may manifest itself as continuous replication and spread of virus, which may lead to disease (e.g. as occurs in SSPE), or the virus may exist in a quiescent state but from which it may be occasionally reactivated. In the former situation large amounts of defective interfering genomes may build up with time and may indeed be essential for the development of the disease, whereas in the latter case defective interfering genomes may not have a role to play. Using simian virus 5 (SV5) as our model system, we have been primarily concerned with the possibilities of the latter type of persistent infection. Specifically our longer term aims are to determine: (i) whether paramyxovirus infections can indeed be established in which the virus genome remains transcriptionally inactive, (ii) the molecular mechanisms involved in the establishment and maintenance of such infections, and (iii) why the immune system apparently fails under certain circumstances to clear paramyxovirus infections.

The virion of SV5 contains at least six structural proteins (Mountcastle et al., 1971; McSharry et al., 1975; Peluso et al., 1977). The nucleoprotein (NP), phosphoprotein (P), large (L) protein and possibly the V protein, together with the single-stranded genomic RNA make up the nucleocapsid (McSharry et al., 1975; Buetti & Choppin, 1977; Lamb & Paterson, 1991), which is surrounded by a lipoprotein envelope through which the haemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins protrude. The matrix protein (M) is required for the integrity of the virus particle and is located on the inner surface of the envelope. SV5 also specifies a small hydrophobic protein (SH) which, although located in the plasma membrane of infected cells, appears to be non-structural (Hiebert et al., 1985).

Like other paramyxoviruses, SV5 has a genome of negative polarity and thus has to generate three different RNA products during its infectious cycle, namely mRNAs, full-length antigenomic RNA and genomic RNA. The viral polymerase responsible for mRNA synthesis is thought to enter the template at the 3' end, in the non-coding leader sequence and to synthesize sequentially the NP, P/V, M, F, SH, HN and L mRNAs by terminating and restarting at each of the gene junctions. Distinct polar effects on transcription have been observed, the NP mRNA being the most abundant species and L mRNA being the least abundant. This is thought to occur because the polymerase binds to the genomic RNA at a position in the 3' leader sequence but has an increasing chance of disengagement the further it proceeds along the genome during transcription (reviewed in Galinski & Wechsler, 1991).

Although SV5 was first isolated from a culture of monkey kidney cells (Hull et al., 1956), it is most notable as a causative agent of upper respiratory tract infections in dogs and as such is often referred to as canine parainfluenza virus (McCandlish et al., 1978). There are few antigenic or sequence differences between SV5 isolated from simian, human or canine sources (Randall et al., 1987; Baty et al., 1991) and it has been suggested that the virus is probably transferred to monkeys via human contact (Tribe, 1966). Studies on the epidemiology of the virus are complicated by the fact that it is often found as a low-level contaminant of tissue culture cells. This and evidence that SV5 is regularly recoverable from kidney cell cultures of monkeys long after the monkeys have been infected (Tribe, 1966; Atoynatan & Hsiung, 1969; Hsiung, 1972), suggest that this virus is readily able to establish persistent infections both in vivo and in vitro. Here, we examine the infection of mouse fibroblastic cells in tissue culture and present evidence that non-defective virus may reside in cells in a non-replicating and transcriptionally inactive form in cytoplasmic inclusion bodies and suggest one possible mechanism by which paramyxoviruses may avoid elimination by the host's immune surveillance.

Methods

Cells and viruses. Vero cells (Flow laboratories), of a Vero cell line persistently infected with a canine isolate of SV5 termed CP1 (which has been passaged more than 100 times following the initial infection with CP1; Baumgärnter et al., 1987) and BALB/c fibroblasts (BF cells; cloned from a primary cell culture of a BALB/c mouse embryo) were grown as monolayers in 25 cm² or 75 cm² tissue culture flasks, in Dulbecco's modification of Eagle's MEM supplemented with 10% newborn calf serum. All cell lines were negative for mycoplasmas as screened staining with 4,6-diamidino-2-phenylindole (DAPI). The strain of SV5, designated LN (isolated by co-cultivation of human bone marrow cells with a permissive cell line; Goswami et al., 1984) was grown and titrated under appropriate conditions in Vero cells using medium containing 2% newborn calf serum (Randall et al., 1987). In all experiments described, cell monolayers were infected at a multiplicity of 5 p.f.u. of SV5 per cell. The virus inoculum was allowed to adsorb for 2 h after which it was replaced with fresh tissue culture medium containing 2% newborn calf serum.

Antibodies. A detailed description of the monoclonal antibodies (MAbs) and their nomenclature has been given elsewhere (Randall et al., 1987).

Preparation of radiolabelled antigen extracts, immunoprecipitation and SDS-PAGE. BF cell monolayers in 25 cm² tissue culture flasks were infected as described above. At various times post-infection (p.i.) the cells were radioactively labelled for 2 h with L-[35S]methionine (> 1000 Ci/mmol; Amersham) in tissue culture medium containing one-tenth of the normal concentration of methionine (1-5 mg/l). At the end of the labelling interval, the cells were washed in ice-cold PBS and lysed into the immunoprecipitation buffer [10 mm-Tris-HCl pH 7.2, 5 mm-EDTA, 0.5% NP40, 0.65 m-NaCl and 0.1% SDS; 4 x 10⁶ to 6 x 10⁸ cells per ml of buffer] by sonication with an ultrasonic probe. Soluble antigen extracts were obtained after pelleting the particulate material from the total cell antigen extracts by centrifugation at 400000 g for 1 h. Immune complexes were formed by incubating 1 ml
samples of the soluble antigen extracts with an excess of antibody (a pool of MAbs to the HN, F, P, M and NP proteins; 1 µl of undiluted ascitic fluids each of SV5-HN-4a, -F-1a, -NP-a, -P-K, -M-n; Randall et al., 1987) for 2 h at 4 °C. The immune complexes were isolated on an excess of a fixed suspension of the Cowan A strain of Staphylococcus aureus [20 µl of a 10% (w/v) suspension per µl of ascitic fluid for 30 min at 4 °C; Kessler (1975)]. The proteins in the immune complexes were dissociated by heating (100 °C for 5 min) in gel electrophoresis sample buffer (0.05 M-Tris pH 7.0, 0.2% SDS, 5% 2-mercaptoethanol and 5% glycerol), and analysed by electrophoresis through 15% SDS-polyacrylamide gels cross-linked with N,N-diallyltartardiamide (DATD). After electrophoresis gels were fixed, dried and stained with Coomassie blue R-250, and labelled polypeptides were visualized by autoradiography.

**Western blot analysis.** Samples to be analysed were suspended in the appropriate volume of gel electrophoresis sample buffer, boiled for 5 min and analysed in 15% SDS-PAGE cross-linked with DATD. Separated polypeptides were transferred onto nitrocellulose using a semi-dry gel electrophlotter (LKB). For Western blot analysis any unoccupied protein-binding sites on the nitrocellulose were blocked with 20% Marvel/PBS before the filters were incubated with the appropriate MAbs. Bound antibody was detected either with ['2P]-labelled Protein A (Amersham) and autoradiography or with horse-radish peroxidase (HRP)-conjugated Protein A and enhanced chemiluminescence (ECL) detection (Amersham).

**ECL-based assay used to quantify the amount of virus antigens in cell extracts.** At various times following infection, BF cell extracts were prepared by scraping the monolayer into 1 ml of PBS, followed by sonication with an ultrasonic probe. Doubling dilutions of the cell extracts were made in PBS in 96-well microtitre plates. Samples of these dilutions were bound to nitrocellulose filters by sandwiching sheets of nitrocellulose between Terasaki plates, into which different dilutions of cell extract had been placed (10 µl per well) for 30 min. After washing extensively in PBS, the protein-binding sites on the nitrocellulose were blocked by reaction with a suspension of 20% Marvel/PBS for 1 h. Individual sheets of nitrocellulose were then incubated with MAbs specific for the HN, F, P (not V), M or NP proteins, washed thoroughly with PBS and reacted with HRP-conjugated Protein A. Bound Protein A was visualized by ECL (Amersham).

**RNA extraction and Northern (RNA) blot analyses.** Total cell RNA was extracted from infected cells at various times p.i. by lysing 107 cells with 4 M-guanidinium thiocyanate and then separating the RNA by centrifugation through caesium trifloroacetate using the method described by Chirgwin et al. (1979). Fifteen µg of RNA from each time-point was separated by electrophoresis through a 1% agarose gel which contained 2.1 M-formaldehyde and transferred to Hybond-N (Amersham) as described by Sambrook et al. (1989). Plasmid-derived cloned cDNA inserts specific for the HN and P genes (Paterson et al., 1984), were gel-purified and random-primed in the presence of [³²P]dCTP using a T7 Quickprime kit (Pharmacia), and used as probes to detect viral mRNA sequences. An actin-specific oligonucleotide probe, end-labelled with ['³²P]ATP, was used as a control for cellular RNA levels. Detection of negative-sense RNA was achieved using a labelled ssDNA molecule that had been generated in a unidirectional PCR reaction (modified from Sturzl & Roth, 1990). Hybridization was carried out at 60 °C for 20 h in 6 x SSC, 5 x Denhardt's solution, 5% SDS and 250 µg/ml calf thymus DNA in a total volume of 25 ml. Following hybridization, the filter was washed twice with 2 x SSC, once with 2 x SSC containing 0.2% SDS and once with 0.2 x SSC, for 20 min per wash at 60 °C and then exposed to X-ray film. The same filter was used for each probe by stripping in 0.005 M-Tris pH 8.0, 0.002 M-EDTA pH 8.0, 0.1 x Denhardt's solution for 1 to 2 h at 65 °C and then rehybridizing.

**Immunofluorescence.** BF cells and Vero cells to be stained for immunofluorescence were grown on coverslips in Linbro plates with 2 × 105 cells seeded per well. The cells were treated and stained with specific MAbs as described in detail elsewhere (Randall & Dinwoodie, 1986). Briefly, monolayers were fixed with 5% formaldehyde, 2% sucrose in PBS for 10 min at 20 °C, permeabilized with 0.5% NP40, 10% sucrose in PBS for 5 min at 20 °C and washed three times in PBS containing 1% calf serum. For detection of the P protein, cells were stained by direct immunofluorescence using a pool of MAbs (SV5-P-a to-k) that had been conjugated with fluorescein isothiocyanate (FITC). The other virus proteins were detected using indirect immunofluorescence. Cells were first reacted with the appropriate SV5-specific MAbs (as ascitic fluids diluted 1:100 in PBS with 1% newborn calf serum) before being reacted with Texas Red-conjugated anti-mouse Ig antibody. In most situations, the cells were first stained for a specific virus protein using the indirect method and then counter-stained for the P protein using FITC-labelled anti-P antibodies. DNA was stained by adding a fluorescent DNA-binding dye (DAPI, 20 µg/ml) to the Texas Red-conjugated anti-mouse antibody.

**Cloning of cell lines persistently infected with SV5.** Monolayers of BF cells were infected with 5 p.f.u. of SV5 per cell. At 4 h p.i. the cells were trypsinized to make a single-cell suspension. Various dilutions of the cell suspension were made such that after seeding there were less than five colonies growing per 96-well microtitre plate. In addition, within 3 days of plating, the 96-well microtitre plates were checked by microscopy to ensure that only individual colonies were growing in a well. When 50 to 75% confluent, the cells were trypsinized, grown to confluence in 24-well Linbro plates before being used in immunofluorescence studies.

To isolate single colonies of cells from the persistently infected Vero cell line, a similar procedure was used with the modification that during the initial cloning procedure the medium was supplemented with a pool of MAbs (Randall et al., 1987) specific for the HN and F proteins (SV5-HN-1b, -3a, -4a, -5a and -F-1a; 1:100 dilution of ascitic fluid) such that the medium had a neutralizing titre of 1:2000. After seeding of the cells onto coverslips for immunofluorescence, the media was not supplemented with anti-HN and anti-F antibodies.

**Generation of cytotoxic T lymphocytes (CTLs) and cytotoxicity assays.** Spleens were removed from mice that had been infected intranasally with SV5 4 to 8 weeks previously. Spleen cells were isolated and restimulated in vitro with SV5-infected spleen cells for 5 days as previously described for the generation of Tc cells specific for respiratory syncytial virus (Bangham et al., 1985).

Target cells were BF cells that had been infected with SV5 at a m.o.i. of 5 to 10 p.f.u. per cell for 1 or 5 days. Uninfected BF cells were used as controls for non-specific lysis. Single cell suspensions of the BF cell populations were made by trypsinization and the cells were labelled with ⁵¹Cr. A standard ⁵¹Cr release assay was performed in U-bottomed microtitre plates. Tests were set up in triplicate using 10⁴ target cells/well. The percentage lysis was calculated as [(sample c.p.m. - background c.p.m.) / (total c.p.m. - background c.p.m.)] x 100, where total c.p.m. is the radioactivity released by targets treated with 01% SDS.

**Results**

**Infection of BF cells with SV5; general properties**

Infection of BF cells at a high m.o.i. with SV5 did not result in the permissive pattern of infection typically observed in other cell types (e.g., Vero or BHK cells). Instead a restricted infection ensued in which there was
no discernible cell fusion and the majority of cells survived the infection. SV5 did not form plaques on BF cells and there was little or no spread of infectious virus from cell to cell. Also, relatively low levels of infectious virus were produced (\( \geq 1 \) p.f.u./cell), that could be titrated on fully permissive cells, such as Vero cells. In infectious centre assays (in which infected BF cells were co-cultivated with Vero cells), when the BF cells were tested within 1 day following infection, 100\% of the cells acted as infectious centres. The percentage of cells giving rise to infectious centres (measured after 5 days co-cultivation with Vero cells) decreased with time, so that by 5 days p.i. only 20 to 40\% of BF cells acted as infectious centres. However, immunofluorescence studies using MAbs specific for the P or NP proteins, showed that at 5 days p.i. more than 95\% of the BF cells remained infected (Fig. 1; described and discussed in greater detail below). Therefore it appeared that by 5 days p.i., a majority of cells, although infected, did not give rise to infectious centres (possible reasons for this are discussed below). Whether all the cells would have eventually given rise to infectious centres if incubated with Vero cells for a longer period of time, was technically too difficult to examine.

\textbf{Virus protein synthesis and protein stability in BF cells}

To examine further the replication of SV5 in BF cells a time-course of virus protein synthesis was undertaken. This was achieved by metabolic labelling of infected cells at various times p.i., after which the virus proteins were immunoprecipitated using a pool of MAbs specific for HN, F, M, P/V and NP. This analysis showed that although all the virus proteins were synthesized throughout the time-course of infection, there was substantially more ongoing virus protein synthesis at 1 day p.i. than at 5 days p.i. (Fig. 2a) with the most significant reduction occurring between 1 and 2 days p.i. (data not shown). Western blot analysis of the same cell extracts clearly demonstrated that, despite the decrease in protein synthesis, the levels of the P and P\(^*\) proteins remained similar at 1 and 5 days p.i. (Fig. 2b), whereas the amount
SV5 quiescent infections

Fig. 2. (a) Analysis of radioactively labelled polypeptides present in immune precipitates formed by the reaction of a pool of MAbs, specific for the HN, F, NP, P/V and M proteins (SV5-HN-4a, -5a; -F-la; -NP-a; -P-e, -P-k; -M-n, respectively) with soluble antigen extracts made from BF cells that had been infected with SV5 for 1 (lane 1) or 5 (lane 2) days. Cells were labelled with [35S]methionine for 2 h prior to extraction. (b) Autoradiogram of a Western blot in which MAb SV5-P-e was used to detect the presence of P and P-related proteins (P* and P°) in unlabelled total cell extracts of BF cells that had been infected with SV5 for 1 day (lane 1) and 5 days [lane 2; parallel cultures to those used in (a)]. Polypeptides in (a) and (b) were separated by electrophoresis through a 12% SDS-polyacrylamide slab gel.

of the P-related protein, P*, was significantly reduced by 5 days p.i. [for further description of P* and P° see Thomas et al. (1988)]. Similar Western blot analysis, using a MAb that recognizes both P and V, showed that while the amount of P protein stayed constant between 1 and 5 days p.i., the relative level of the V protein decreased (Fig. 3). Furthermore a Western blot analysis comparing the levels of the NP and M proteins, showed that the relative amount of the NP protein was similar at 1 and 5 days p.i. but that the level of the M protein decreased (data not shown).

As there appeared to be a shut-off of the synthesis of all the viral proteins early in infection, it was presumed that the steady-state level of each viral protein was a reflection of its stability in the cells, e.g. the finding that the levels of the NP and P proteins were similar at 1 and 5 days p.i. suggests that these proteins were relatively stable in contrast to the V and M proteins. To confirm the results of the Western blot analysis and to examine the stability of other virus proteins (such as the HN and F proteins for which none of the MAbs reacted in a Western blot), a series of experiments was performed in which total cell extracts were made of infected cells at various times p.i. Doubling dilutions of these cell extracts were dot-blotted onto nitrocellulose and the relative levels of the individual virus proteins at different times were estimated by their reaction with MAbs specific for NP, P (not V), M, HN and F proteins (Fig. 4). These results confirmed that the relative amount of NP was constant between 1 and 5 days p.i. In comparison, F appeared extremely unstable and was undetectable by this method after 3 days p.i. As expected from the Western blot analysis, M protein levels also decreased relatively quickly. Surprisingly, HN appeared fairly stable, although less so than NP. A slight decrease in the level of P was also noted, but Western blot analysis suggested that this may be accounted for by the selective degradation of P-related proteins, such as P°. These results suggest that the bulk of virus proteins that could be detected in cells over the time-course, were synthesized in the early stages of infection. However, the levels of some viral proteins (NP and P) remain relatively constant throughout the time-course suggesting that they are stable. The other viral proteins (P*, V, M and F) are degraded more rapidly and thus disappear from the protein pool.

Virus RNA synthesis in BF cells

To ascertain whether the reduction in the levels of virus protein synthesis, observed over time, was a reflection of a decrease in ongoing virus transcription, the levels of selected mRNAs were measured by Northern blot
Fig. 4. Analysis of the relative amounts of the NP, P, M, F and HN proteins in lysates of BF cells infected with SV5 for 1 to 5 days (columns 1 to 5 respectively). Doubling dilutions of total cell extracts were bound to nitrocellulose and the respective antigens detected with pools of specific MAbs (SV5-NP-a; SV5-P-a, -b, -c, -e, -j; SV5-M-a, -b, -c, -f, -h; SV5-F-1a; SV5-HN-1b, -4b, -5c, -5d, -5e). Bound antibody was detected in an ECL reaction following the reaction of the nitrocellulose with HRP-conjugated Protein A.

Fig. 5. (a) Northern blot analysis of total RNA extracted from BF cells infected with SV5 for 1, 3 and 5 days (as shown above lanes) probed using 32P-labelled random-primed cloned gene probes specific for the P gene, HN gene and an actin-specific oligonucleotide probe, end-labelled with [α-32P]ATP. The same filter was used for each probe by stripping and then rehybridizing. (b) A dot blot of the same RNA extracts that were used for the Northern blot described in (a), probed using a 32P-labelled positive-sense DNA probe to detect genomic-sense RNA levels. The filter was stripped and rehybridized with an actin-specific oligonucleotide probe. Lanes U represent uninfected cells.

analysis. Fig. 5 clearly demonstrates that the relative mRNA levels of both the P and HN genes are significantly reduced at 3 and 5 days p.i. as compared to 1 day p.i. This was not because of a general reduction in the overall levels of mRNA synthesis as the levels of actin mRNA were similar at each time. To investigate the levels of genomic-sense RNA present in the cell population, doubling dilutions of the same total cell RNA were spotted onto nylon and probed with a 32P-labelled oligonucleotide specific for negative-sense RNA. These results demonstrate clearly that the levels of genomic RNA remain constant throughout the time-course but that mRNA levels are significantly reduced by 3 days p.i., probably accounting for the reduction in viral...
protein synthesis at later times in infection. The dot blot results showing continuing high levels of viral RNA were confirmed by Northern blot analysis of RNA extracted from BF cells at 1 and 3 days p.i. (Fig. 6). This demonstrated that the genomic/anti-genomic RNA was of the expected Mr and that there was no evidence for the accumulation of defective genomes.

Examination by immunofluorescence of the levels of expression and distribution of virus proteins in individual cells

The results presented above show that, with time, there is a marked decrease in viral transcriptional activity and protein synthesis in the infected cell population. However, these analyses do not distinguish between a general down-regulation of viral transcriptional activity in all the infected cells and, alternatively, a situation in which the virus is transcriptionally inactive in the majority, but active in a minority of infected cells. To examine the state of the virus in individual cells, immunofluorescence analysis of infected cells at different times p.i. was undertaken, using a panel of MAbs specific for the HN, F, NP, P/V and M proteins. Fig. 1 shows a typical example of cells at 1 and 5 days p.i. stained for the viral proteins P and F and for cellular DNA. A number of salient observations arose from these studies. (i) At 1 day p.i. all the virus proteins could be detected by immunofluorescence in the majority of infected cells. (ii) By 5 days p.i. while the majority of cells were still positive for the P and NP proteins a significant proportion of these cells were only weakly positive or were negative for HN, F and M proteins (Fig. 1). (iii) The distribution of the NP and P proteins clearly altered with time p.i. At 1 day p.i., although some protein was beginning to aggregate in the cytoplasm, these proteins were primarily found distributed evenly through the cytoplasm, giving diffuse fluorescence. Small amounts of P and NP were also located, together with the HN, F and M proteins, in long filamentous structures protruding from the cell surface (not shown). However, by 5 days p.i. in the majority of cells, the NP and P proteins co-localized in large cytoplasmic inclusion bodies (Fig. 7) from which the HN, F and M proteins were excluded (Fig. 1). (iv) At 5 days p.i. the infected population had become heterogeneous, i.e. while the majority of cells were clearly positive for only NP and P there was a small proportion of cells which resembled those at 1 day p.i. in that all the viral proteins were readily detectable. Furthermore, at 5 days p.i. in cells in which there was no diffuse cytoplasmic fluorescence of the NP and P proteins, but in which there were clear cytoplasmic inclusion bodies containing these proteins, there was no, or very little F, HN or M present.
The immunofluorescence results thus supported the Western blot (Fig. 2 and 3) data and the comparative analysis of the relative amounts of the different virus proteins at 1 to 5 days p.i. (Fig. 4) in that the NP and P proteins could be detected in the cells throughout the time-course, whereas the M, F and HN clearly disappeared from the majority of cells with time. Thus although the HN protein was more stable than the F and M proteins, it did not appear to be as stable as the NP or P proteins since, at later times p.i. there were many cells positive for NP and P but negative for HN. It is not clear whether the stability of the NP and P is an intrinsic property of these proteins or whether it results from their aggregation into inclusion bodies. What is clear is that either all the NP and P proteins made must aggregate, or that those which do not must be degraded, because at later times p.i. the majority of cells have large inclusion bodies but no sign of diffuse cytoplasmic fluorescence.

Given the instability of F and M, those cells which remained positive by fluorescence for these proteins at later times presumably represented a small population of cells in which virus synthesis was still, or recently, active. This subpopulation could therefore account for the low overall level of viral transcription and protein synthesis that was still ongoing at 5 days p.i. Conversely, in the majority of cells, in which only NP and P were detected, virus transcription and protein synthesis would either be greatly reduced or absent (Fig. 2 and 5).

**Single cell cloning of infected cells**

The above results suggest that at later times p.i. a high proportion of cells that contained inclusion bodies were not actively synthesizing virus proteins. As the viral RNA remains at relatively high levels throughout the 5 day period, in the face of diminished levels of viral transcription and protein synthesis, it seems plausible that these inclusion bodies contain aggregates of inactive virus nucleocapsids. Alternatively, if there was ongoing virus replication, or if active nucleocapsids were distributed evenly throughout the cytoplasm, in all virus-infected cells, it would be expected that when a cell divides both daughter cells would be infected. Immunofluorescence data suggested that the inclusion bodies may not segregate equally upon cell division (Fig. 8), and thus, if there was no ongoing replication, it might be expected that only some daughter cells would become infected. Indeed, following passage of infected BF cells there was an obvious reduction in the proportion of cells that appeared infected as judged by immunofluorescence (data not shown).

To determine whether or not all daughter cells became infected upon division, BF cells were cloned in 96-well microtitre plates immediately following infection. Ten independent clones were obtained which were examined for the presence of virus by immunofluorescence. Positive fluorescence was observed in five clones and in these clones only a small proportion (1 to 5%) of cells were infected. Since the cell initially cloned must have been infected (in the virus-positive clones at least), these results demonstrate clearly that upon cell division not all the daughter cells become infected. Of the infected cells in the different clones, some were positive for all the virus proteins (Fig. 9a) while others were positive for only the NP and P proteins (Fig. 9b), suggesting that in BF cells SV5 may flux between an active and an inactive state.

As the M, HN and F proteins could be detected in some of the cloned cells, it was speculated that in these cells there may have been ongoing or at least recent virus transcription and replication. If this was the case, it was possible that these cells could act as infectious centres. To test this, virus-positive cell clones were co-cultivated with Vero cells and 3 days later examined by immunofluorescence. Plaques could be seen distinctly, developing in the monolayers, demonstrating the spread of infectious virus into the Vero cell population. The distribution of each of the virus proteins, including glycoproteins, in these plaques was typical of a normal, productive infection (Fig. 10c). However, individual cells could still be detected in the mixed cultures which although positive for NP and P, remained negative for the other virus proteins (for example HN, Fig. 10a). In such cases there was no evidence for the rescue of virus from the cells. In other situations it appeared that spread of infectious virus was only just beginning and that the original, infected BF cell could still be observed (Fig. 10b). Culture medium taken from these mixed cultures (prior to them being fixed for immunofluorescence), was found...
to contain infectious virus that gave rise to normal plaques on Vero cell monolayers (data not shown).

Virus inclusion bodies could also be detected in BF cells that had not been cloned but had been passaged 10 times (50+ cell divisions) over a 12-week period (after which time the cells were no longer kept). Although, by this time >95% of the cells showed no sign of infection, it was still possible to rescue low levels of infectious virus from these cells by co-cultivating them with Vero cells. The re-isolated virus did not appear to be adapted for growth in BF cells and showed the same pattern of infection as seen with the original virus (data not shown).

Sensitivity of SV5-infected BF cells to cell-mediated lysis

Given that in a population of BF cells at 5 days p.i., protein synthesis is continuing only at low levels or is absent in most of the cells and that in these cells P and NP appear to be stable, it was of interest to study the sensitivity of this cell population to immune attack compared to a cell population at the earliest stages of infection, in which virus protein synthesis is ongoing at a high level in the majority of cells. Fig. 11 shows a typical result of an experiment in which splenocytes, isolated from BALB/c mice previously infected with SV5, were used to lyse BF cells at 1 and 5 days p.i. From these experiments it was clear that BF cells at 5 days p.i. were less sensitive to lysis than those at 1 day p.i., although at 5 days p.i. >95% of cells appeared positive, by immunofluorescence, for the NP and P antigens. This result was obtained in four independent experiments. No attempt was made in these experiments to determine whether the lysis observed was due to class I, class II or natural killer (NK) cells. However, P815 cells (BALB/c mastocytoma cells that only express class I major histocompatibility complex molecules and that are relatively insensitive to NK activity) infected with SV5 for 1 day were also more sensitive to lysis than those which had been infected for 5 days (60% and 25% ¹¹Cr release respectively; data not shown).

Examination of Vero cells persistently infected with SV5

Having demonstrated that upon cell division of infected BF cells not all daughter cells may become infected, it

Fig. 9. Photographs of the distribution of the HN and P proteins in monolayers of cloned infected BF cells (see text). Less than 5% of the cells were positive by immunofluorescence for the P protein; those cells were either weakly positive or negative for HN. In those cells which were positive for P but negative for HN, the P protein was usually confined to single cytoplasmic foci (b, arrow). Monolayers were fixed and stained with a MAb specific for the HN protein using indirect immunofluorescence before being counter-stained with a mixture of FITC-conjugated MAbs specific for the P protein.
was of interest to determine whether such a situation could also occur in cells capable of supporting normal virus replication. To examine this question we were kindly provided by Dr Baumgartner (Institut für Veterinär-Pathologie, Universität Giessen, Germany) with a Vero cell line persistently infected with a canine isolate of SV5, termed CPI+. This cell line had been passed more than 100 times following the initial infection with the virus (Baumgärtner et al., 1987). Examination of the cells by immunofluorescence using MAbs specific for the NP, P/V, M, HN and F proteins demonstrated that while the majority of cells (> 90%) were positive for NP and P a small proportion of cells appeared negative for all virus proteins (data not shown). Furthermore, most of the cells positive for NP and P were negative for the other virus proteins. In those cells positive for the NP and P proteins it was also clear that many of them appeared to have large cytoplasmic inclusion bodies similar to those observed in BF cells. Little or no infectious virus was released into the culture medium of

Fig. 10. Photographs of the distribution of the P and HN proteins in three different fields of view of monolayers of Vero cells co-cultivated for 3 days with the infected BF clones described in Fig. 7. In (a) there appears to have been no spread of infectious virus from the infected BF cell (arrowed), in (b) it can be seen that virus is just beginning to spread into the Vero cell population, and in (c) a clear plaque has developed in the monolayer. Monolayers were fixed and stained with a MAb specific for the HN protein using indirect immunofluorescence before being counter-stained with a mixture of FITC-conjugated MAbs specific for the P protein.
the persistently infected cells (> 10 p.f.u./ml), but infectious virus could be recovered following co-cultivation of these cells with uninfected Vero cells. Consequently some non-defective genomes must have remained present in these cells.

The persistently infected Vero cells were cloned in the presence of a pool of neutralizing MAbs specific for the HN and F glycoproteins to prevent spread of the infection via any small amounts of infectious virus that may have been released into the culture medium or through cell–cell fusion. Of 11 clones isolated there were marked differences in the percentage of cells infected, ranging from 0 to 100% (Fig. 12). Only in one cell line were all the cells positive for the NP and P proteins. Furthermore, of the cells positive for NP and P immunofluorescence there were differences in the number that were also positive for the other virus proteins. For example, a cell line was obtained in which approximately 50% of cells were positive for NP and P but none of the cells were positive for HN (Fig. 12a). On the other hand, another cell line which had approximately 80% of cells positive for NP and P about half of these were positive for NP and P but none of the cells were positive for HN (Fig. 12b). Cells that were negative by immunofluorescence for all virus proteins could subsequently be infected with infectious virus (data not shown). However, it was not possible to superinfect cells that were positive by immunofluorescence (data not shown). Because of the high passage number of the cells, the situation was obviously complicated by the presence of defective genomes. However, these results again suggested that in a proportion of cells the virus genome may not be undergoing active transcription or replication at the time of division; otherwise all the cells in the cell clones should have remained infected.

Discussion

The results that stimulated this analysis came partially from studies on the immune response to SV5 in mice. Following intranasal infection of BALB/c mice with SV5, increasing amounts of the P protein and nucleic acids could be detected in their lungs (by Western blot and in situ hybridization analyses respectively) until 3 days p.i. (Randall et al., 1988). Thereafter, in the absence of an active immune response, the virus load within lungs remained relatively constant (Young et al., 1990; Randall & Young, 1991). A possible explanation for these results was that, following an initial wave of virus transcription and replication, the virus became transcriptionally inactive, with the bulk of the P protein made early in infection remaining in a stable form in infected cells. The results presented here, on the infection of BF cells in tissue culture, support this view. Thus it appears that following infection of BF cells with SV5, there is an initial wave of virus RNA and protein synthesis. During this phase all the virus proteins can be detected in infected cells by immunofluorescence. At later times the small amount of ongoing virus protein synthesis probably occurs in cells which are positive for all the virus proteins. In those cells that are positive for NP and P proteins but negative for the other virus proteins, it is likely that the NP and P proteins that can be detected were made at earlier times but remained stable in cytoplasmic inclusion bodies. In contrast, other virus proteins, such as HN, F, M, V and the P-related protein P*, were less stable and were degraded with time. In this scenario, at later times the majority of virus genomes would be unavailable for transcription or replication since they would be trapped in nucleocapsids aggregated in stable cytoplasmic inclusion bodies. However, in some cells with these inclusion bodies, it is possible that the virus may subsequently have become active, possibly because of the release of infectious nucleocapsids from the inclusion bodies into the surrounding cytoplasm. This could account for the small proportion of infected cloned cells that express all the virus proteins and also for the capacity to re-isolate infectious virus.

An alternative explanation for these results is that in some infected BF cells the virus continues to replicate (and it is from these cells that infectious virus can be rescued), while the majority of cells somehow manage to 'cure' themselves of the infection. However, since the BF cells themselves had been cloned from a single cell, it is difficult to understand why a small percentage of cells should continue to replicate virus while others did not. It could be argued that mutations in the virus genome might result in continuous virus replication in a proportion of dividing BF cells. However, if this was the case, then upon cloning infected BF cells, we should...
Fig. 12. For legend see opposite.
have been able to isolate clones in which all the cells were infected, but this was not done. Furthermore, given that all the virus proteins can be made to high levels in BF cells, if there had been constant ongoing transcription and replication it should have been relatively easy to isolate virus capable of spreading within the BF cells, either following cloning of infected cells or on passage of uncloned infected cells. However despite numerous attempts, we failed to isolate mutant viruses that would grow to high titres in BF cells. This would be expected if the re-isolated virus had remained quiescent in inclusion bodies prior to its reactivation. Thus, we believe the most likely explanation for the results presented here is that in BF cells SV5 fluxes between an inactive and active state. Even in persistently infected Vero cells (in which defective interfering particles undoubtedly have a role to play in establishing the persistent infection) there is good evidence that not all the virus genomes may be actively replicated or transcribed in all cells at a given time. Thus, upon cloning a persistently infected Vero cell population which had been passaged over 100 times, not all the cells in any given clone remained infected (Fig. 12). Again the most likely explanation for being able to isolate clones in which only a small percentage of cells remain infected is that upon division of these cells not all of the cells may have been actively replicating the virus at any one time, otherwise all daughter cells should have remained infected.

In BF cells there appeared to be some block in the production of infectious virus, in that at 1 day p.i., although there are similar levels of virus protein and viral RNA synthesized compared to that seen in permissive cells, little infectious virus is released. The absence of cell–cell fusion in BF cells and the production of subnormal amounts of infectious virus cannot be explained simply by incorrect processing of the fusion protein since \( F_0 \) was cleaved to \( F_1 \) and \( F_2 \) (Fig. 3). Electron microscopic studies have shown large aggregates of nucleocapsids accumulating in the cytoplasm of BHK-21-F cells infected with SV5. These cells also produced little infectious virus and it was suggested that there may be some block in virus maturation (Compans et al., 1964). In fact any block in virus maturation (e.g. through antibody-induced antigenic modulation; Fujinami & Oldstone, 1979, 1980) may result in the accumulation of nucleocapsids, the formation of cytoplasmic inclusion bodies, the down-regulation of virus transcription and replication and the survival of the cell. Interestingly, the cytoplasmic inclusion bodies seen in cells persistently infected with SV5 appear similar to aggregates seen in diseased tissue in which a persistent parainfluenza virus infection is known or suspected, e.g. SSPE and Paget's Bone Disease (Harvey et al., 1982; Iwasaki & Koprowski, 1974; Mills & Singer, 1976; Raine et al., 1974; Rebel et al., 1977).

Cell-mediated immune responses (including class I-restricted T cells) play a critical role in the recovery from many parainfluenza virus infections (reviewed in Randall, 1993). During virus transcription, a small proportion of newly synthesized virus proteins will be proteolyzed in the cytosol of the infected cells and the resulting peptides processed for presentation to class I-restricted T cells (reviewed in Hanke & Randall, 1994). Thus, when considering the establishment of persistent infections in vivo, one question that has to be considered is why the cell-mediated immune response might fail under certain circumstances to clear the infection. If during the initial parainfluenza virus infection, when CTLs are absent or in limited numbers in infected tissues, a proportion of infected cells survive the infection (perhaps because of the cell type, its differentiation or physiological state, or through action of interferon), then given the scenario outlined above, it is possible that in a small proportion of cells, the virus genome would become transcriptionally inactive in cytoplasmic inclusion bodies. If such inclusion bodies are reasonably stable to proteolytic degradation and no ongoing virus protein synthesis occurs, then such cells may not be recognized by virus-specific CTLs (as is suggested by results presented here). The virus may remain inactive for prolonged periods of time (weeks, months or possibly even years) but occasionally may be activated to initiate another round of virus replication. If this occurred at a time when there was no ongoing illness, local immunity may not prevent a limited spread of infection. Individuals in which this occurred may then release small amounts of infectious virus into the environment, possibly without developing overt disease because of the rapid secondary immune response. If this does indeed occur naturally, then the presence of individuals within a community capable of shedding...
infectious virus over a prolonged period of time, might influence the epidemiology of infection. There is some evidence for this (reviewed in Randall & Russell, 1991). For example, in a study of an isolated community at the American Antarctic Research Station, Muchmore et al. (1981) showed that healthy asymptomatic individuals could shed parainfluenza virus type 3 over a 6 to 8 month period. Furthermore, they concluded that two outbreaks of respiratory illness occurring within that 8 month period could best be explained as being initiated by persistently infected individuals.

There are obvious selective advantages for a virus if a significant proportion of infected individuals secreted infectious virus over a prolonged period of time. If this was the case for paramyxoviruses, it might be expected that specific molecular mechanisms would have evolved that influence the establishment and maintenance of such infections. Indeed, the relative ease with which some paramyxoviruses cause persistent infections suggests that their mode of replication and genome structure may specifically facilitate the establishment of such infections. In this respect, it is of interest to note that the P-related proteins of Sendai virus (V, W and C) have been shown to influence the levels of virus replication and transcription (Curran et al., 1991, 1992). When considering the virus factors that may influence the establishment and maintenance of persistent infections, it may be of relevance that the selection of virus that grows to high titres in tissue culture may actually select against virus which can efficiently establish persistent infections. Furthermore, the ability of human paramyxoviruses to establish persistent infections may have been an extremely important property in the past, when population densities may not have been sufficiently high to maintain viruses unable to become persistent, within small communities. However, with increasing population densities, the requirement of viruses to establish persistent infections may have become less important and indeed some viruses may have lost this quality altogether.

In conclusion, data presented here suggests that paramyxoviruses under certain circumstances may become transcriptionally inactive inside cells and that if this occurs in vivo it may help to explain why such viruses can establish persistent but silent infections. The mechanisms and factors that influence the establishment of such quiescent infections are currently under investigation.

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References


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