Journal of NeuroVirology (1998) 4, 438–444

http://www.jneurovirol.com

Short Communication

Infectious simian varicella virus expressing the green fluorescent protein

R Mahalingam[1], M Wellish[1], T White[1,2], K Soike[4], R Cohrs[1], BK Kleinschmidt-DeMasters[1,3] and DH Gilden[1,2]

Departments of [1]Neurology, [2]Microbiology and [3]Pathology, University of Colorado Health Sciences Center, Denver, Colorado 80262; and [4]Delta Regional Primate Research Center, Tulane University, Covington, Louisiana 70433, USA

Clinical, pathologic, immunologic and virologic features of simian varicella virus (SVV) infection in primates closely resemble varicella-zoster virus (VZV) infection in humans. Such similarities provide a rationale to analyze SVV infection in primates as a model of varicella pathogenesis and latency. Thus, we constructed an SVV-expressing green fluorescent protein (SVV–GFP) by inserting the GFP gene into the unique short segment of the virus genome by homologous recombination. Analysis of recombinant viral DNA and the expressed proteins of plaque-purified SVV–GFP confirmed the location of the GFP insert and that the recombinant SVV expressed the 27 kDa GFP. Infection of monkey kidney cells in tissue culture with SVV–GFP revealed bright green fluorescence associated with the characteristic focal cytopathic effect produced by SVV infection. Microscopic examination of lung from a 3-month-old African green monkey 10 days after infection with SVV–GFP revealed bright green fluorescence in areas of acute necrotizing pneumonitis. SVV–GFP allows ready identification of cells infected with SVV both *in vitro* and *in vivo*, and will be useful for further analysis of varicella pathogenesis and latency in experimentally infected animals – studies not possible in humans.

Keywords: simian varicella virus; green fluorescent protein

Introduction

Varicella-zoster virus (VZV) causes chickenpox (varicella) in children, becomes latent in dorsal root ganglia, and reactivates decades later to produce shingles (zoster). Zoster and its neurologic complications are an important cause of morbidity, and occasionally mortality, particularly in the elderly and immunocompromised populations. Any attempt to prevent virus reactivation must begin with an understanding of the physical state of virus during latency, not the least of which is identification of the cell that harbors virus in latently infected human ganglia, including cells that transport virus throughout the nervous system.

Such studies require an animal model and a virus marker. The animal model is provided by simian varicella virus (SVV) infection of primates in which clinical (Padovan and Cantrell, 1986), pathological (Dueland *et al*, 1992), immunological (Felsenfeld and Schmidt 1977) and virological (Clarke *et al*, 1992) features, including latency (Mahalingam *et al*, 1991) resemble VZV infection in humans. The virus marker is green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, which emits bright green fluorescence at 509 nm (Chalfie *et al*, 1994). GFP is stable with minimal photobleaching (Chalfie *et al*, 1994), and gene encoding GFP has been modified to enhance the fluorescence several-fold (Zolotukhin *et al*, 1996). We inserted the GFP gene into the unique short segment of the virus genome, and observed SVV-infected cells *in vitro* and *in vivo* for green fluorescence.

Results and Discussion

Detection of green fluorescence in cells infected with SVV–GFP

After transfection with the recombinant clone containing RSV–GFP (Figures 1 and 2), we observed bright green fluorescence associated with a focal cytopathic effect in SVV-infected BSC-1 cells. These foci were purified by passaging through monolayers of BSC-1 cells. Fluorescence detected after infection with purified SVV–GFP is shown in Figure 3. Most if not all fluorescent cells were SVV-

Correspondence: R Mahalingham
Received 21 October 1997; revised 21 November 1997; accepted 12 December 1997

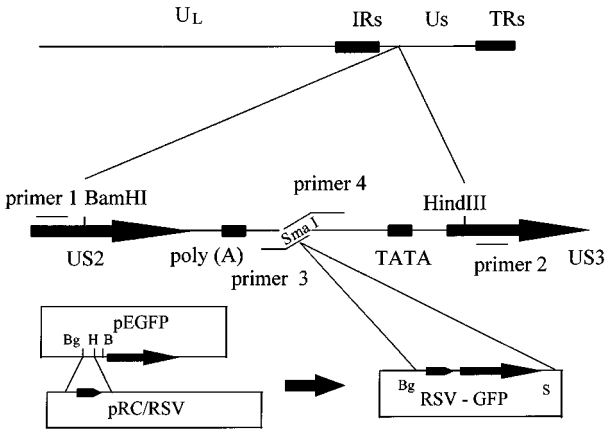

Figure 1 SVV DNA construct containing Rous sarcoma virus RSV–GFP in the U_S segment. The location of the unique long (U_L) and unique short (U_S) segments bounded by internal and terminal repeat sequences (IR_S and TR_S), the US2 gene, the poly(A)-addition site for US2, the putative TATA box for the US3 gene, US3, and primers 1, 2, 3 and 4 are indicated. The *Sma*I site at the 3′- and 5′-ends of primers 3 and 4 is shown. pEGFP and pRC/RSV vectors with the location of *Bgl*II (Bg), *Hind*III (H), *Bam*HI (B) and *Ssp*I (S) restriction sites are also identified. The DNA fragment containing RSV–GFP was cloned into the *Sma*I site.

positive when analyzed using rabbit anti-SVV-antiserum (data not shown). We did not observe any fluorescence in cells infected with the wild-type SVV (data not shown).

Characterization of SVV–GFP

We infected BSC-1 cells with plaque-purified SVV–GFP. We used total DNA extracted from SVV–GFP- and SVV-infected BSC-1 cells in PCR-amplifications using SVV-specific primers located outside the RSV–GFP insert (Figure 4). Upon hybridization using a SVV-specific internal oligonucleotide, we detected 1857 bp and 258 bp fragments in DNA from SVV–GFP- and SVV-infected-BSC-1 cells, respectively. The 258 bp fragment was absent in DNA from SVV–GFP-infected cells even after 24 h of exposure on phosphoimager (Molecular Dynamics, Sunnyvale, California). The difference in size between the two bands accounted for the 1599 bp RSV–GFP insert in the SVV genome and confirmed its insertion at the desired location.

We also extracted total protein from uninfected BSC-1 cells, BSC-1 cells infected with SVV, and BSC-1 cells infected with plaque-purified SVV–

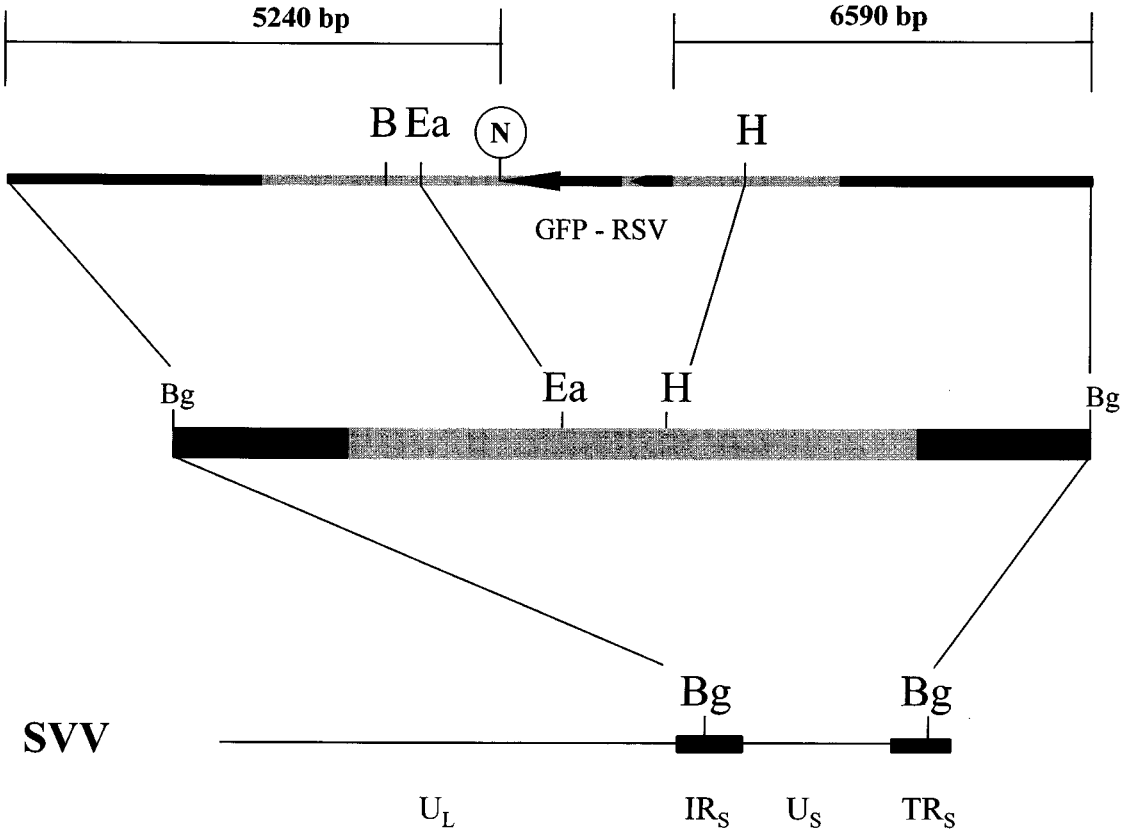

Figure 2 Extension of SVV sequences flanking RSV–GFP. The location of various segments of the SVV genome, the SVV *Bgl*II-D fragment, and the *Bgl*II (Bg), *Eag*I (Ea), *Bam*HI (B) and *Hind*III (H) restriction sites, including the *Not*I (N) site within the RSV–GFP insert are indicated. the *Not*I site was mutated and replaced the *Eag*I–*Hind*III fragment in the SVV *Bgl*II-D recombinant clone with the *Eag*I–*Hind*III fragment containing the RSV–GFP insert. The final 13.5 kb recombinant clone contained RSV–GFP sequences flanked by 5240 and 6590 bp of SVV sequences.

GFP, and analyzed them by Western blotting using rabbit antibodies to GFP (Figure 5). We detected a substantial amount of the 27 kDa GFP only among proteins extracted from SVV–GFP, but not in proteins extracted from uninfected BSC-1 cells, or from SVV-infected BSC-1 cells. Faint bands smaller than 27 kDa seen in Figure 5 are probably due to GFP degradation.

Detection of GFP expression in monkey lung

Our histopathologic examination of monkey lung infected with SVV–GFP revealed extensive necrosis, inflammation and loss of alveoli (Figure 6). Bright green fluorescence, evident in areas of acute necrotizing pneumonitis, indicated productive infection by SVV expressing GFP. Further, we detected SVV-specific antigens in the same area with anti-SVV-antisera (Figure 6). The degree of inflammation and distribution of SVV antigens were similar to that seen in monkey lung after infection with wild-type SVV (Dueland *et al*, 1992), indicating that inflammation was not increased due to GFP expression.

Overall, we showed that SVV–GFP allows ready identification of cells infected with SVV *in vitro* and *in vivo*. The technique will be useful for analysis of varicella pathogenesis as well as to determine the cell-type latently infected with virus in experimentally infected animals–studies not possible in humans.

Material and methods

Cells and viruses

We propagated SVV and SVV–GFP by cocultivation of uninfected cells with SVV-infected cells as described (Gilden *et al*, 1982).

DNA manipulations

All DNA manipulations were performed using enzymes according to the manufacturer's instructions.

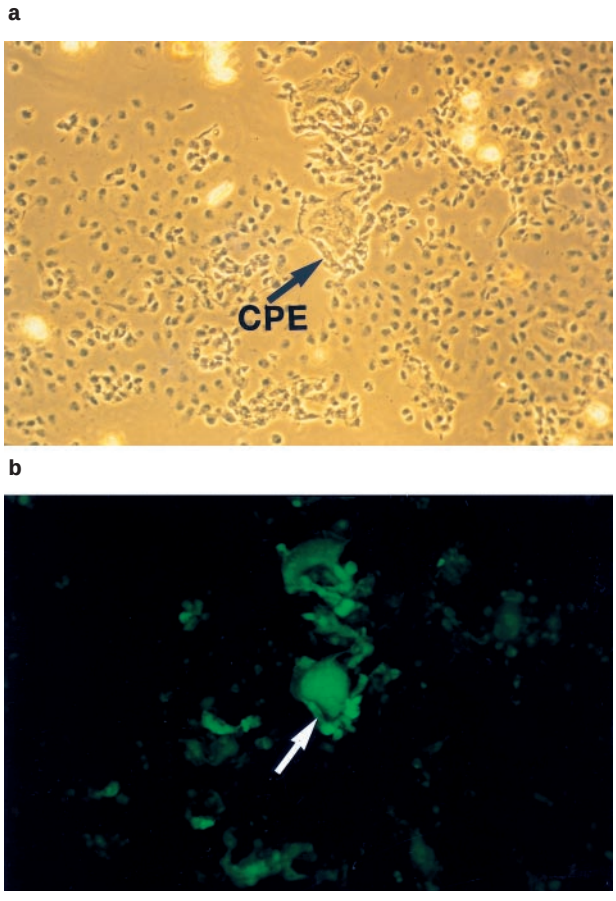

Figure 3 Infection of BSC-1 cells with SVV-expressing GFP. SVV-infected cells in tissue culture were transfected with the 13.5 kb recombinant clone containing RSV–GFP flanked by SVV sequences (Figure 2). (**a**) Black arrow shows the cytopathic effect (CPE) in cells infected with SVV–GFP under normal light. (**b**) White arrow indicates green fluorescence in the same area of CPE shown in panel **a**.

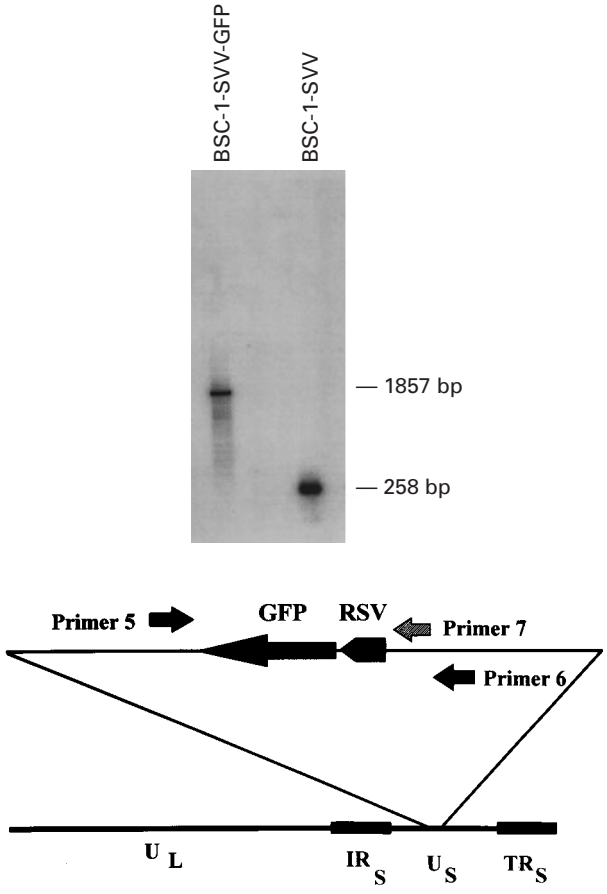

Figure 4 PCR analysis of DNA from SVV–GFP-infected cells. Total DNA extracted from SVV-GFP- and SVV-infected BSC-1 cells was used along with SVV-specific primers 5 and 6 in PCR amplification. The products were analyzed as described in Methods using a ^{32}P-labeled SVV-specific internal oligonucleotide (Primer 7). The location and direction of GFP, RSV, and primers 5, 6 and 7 sequences on the SVV genome and the sizes of the PCR products are indicated.

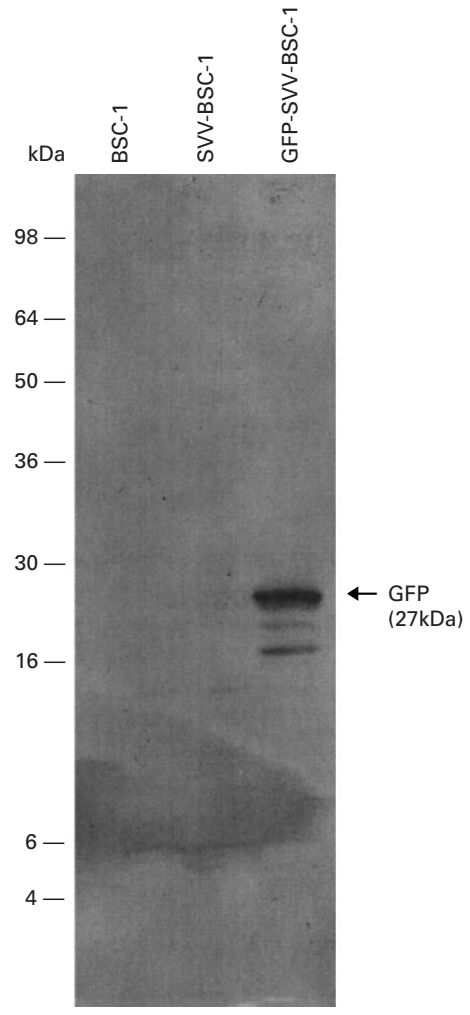

Figure 5 Expression of GFP in SVV-GFP-infected cells. Western blot of total protein from uninfected (BSC-1), SVV-infected (SVV-BSC-1) or SVV–GFP infected (GFP-SVV-BSC-1) cells after incubation with rabbit anti-GFP followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. The 27 kDa GFP was detected only in total proteins from SVV–GFP-infected cells.

Construction of a recombinant clone containing SVV sequences flanking green fluorescent protein (GFP) gene

We selected the intragenic region between SVV genes US2 and US3 (both located in the U_S segment of the SVV genome) to insert the GFP gene (Figure 1). We introduced a *Sma*I site into a DNA fragment containing a portion of the U_S segment of the SVV genome which included a part of the US2 ORF, the poly(A) addition signal for US2, the putative promoter (TATA box) for US3, and part of the US3 ORF (Figure 1). To generate such a DNA fragment, we selected oligonucleotide primers 1, 2, 3 and 4 from the known SVV U_S sequences (Gray *et al*, 1995). We located a *Bam*HI site downstream from primer 1 and a *Hind*III site upstream from primer 2 (Figure 1). We designed primers 3 and 4 such that SVV-specific sequences were attached at their 3′ and 5′-end, respectively, and to an *Sma*I restriction site sequence (GGGCCC). Using DNA from SVV-infected BSC-1 cells with primers 1 and 3, we amplified a 578 bp PCR product; with primers 2 and 4, we amplified a 354 bp fragment. In a third PCR amplification, we mixed the two PCR products (578 and 354 bp fragments) and used the outside primers 1 and 2. The annealing temperature for the third PCR was 40°C for 2 min for seven cycles without any primers followed by 55°C for 2 min for 30 cycles using outside primers 1 and 2. The final 936 bp product had an *Sma*I site at the desired location. We digested the fragment with *Bam*HI and *Hind*III, obtained a 735 bp DNA fragment and cloned it into *Bam*HI–*Hind*III-digested pGem11Zf(+) vector (Promega, Madison, WI). We inserted a 1638 bp DNA fragment containing the RSV promoter (pRC/RSV from Invitrogen, Carlsbad, CA) in front of sequences encoding EGFP (enhanced, red-shifted GFP from Clontech, Inc., Palo Alto, CA) into the *Sma*I site (generated as described above) of the recombinant clone containing the SVV sequences in the pGEM11Zf(+) vector (Promega). The orientation of the insert in the recombinant clone was determined to be *Bam*HI-GFP-RSV-*Hind*III, and the GFP-RSV sequences were flanked by 470 and 270 bp of SVV sequences from the U_S segment of the SVV genome.

Enlargement of flanking SVV sequences around the RSV–GFP insert

To abolish the *Not*I site and a *Eag*I site (part of the *Not*I site) in the recombinant clone described in Figure 1, we digested it with *Not*I (N), blunt-ended with mung bean nuclease (Gibco BRL Inc., Bethesda, MD) and religated. We digested the recombinant clone with *Eag*I and *Hind*III and isolated a 2377 bp DNA fragment containing the RSV–GFP sequences. We digested a recombinant clone containing the 11.9 kb SVV *Bgl*II-D fragment in pGem3Z with *Eag*I and *Hind*III and replaced it with the 2377-bp *Eag*I–*Hind*III fragment containing the RSV–GFP sequences, effectively increasing the size of the SVV sequences flanking RSV–GFP to 5420 and 6590 bp, as shown in Figure 2.

Purification of SVV expressing GFP

We used lipofectin (Gibco BRL) to transfect SVV-infected BSC-1 cells in tissue culture dishes with the recombinant clone containing RSV–GFP. Cells fluorescing green were identified with a fluorescence microscope and transferred onto uninfected BSC-1 cells. We repeated this procedure several times until all cells exhibiting a cytopathic effect were green under UV light, after which SVV–GFP was plaque-purified.

Analysis of DNA from virus-infected cells
Total DNA from SVV-infected and plaque-purified SVV-GFP-infected cells was extracted and 1 ng of the samples were used in PCR reactions using primers 5 and 6. The products were electrophoretically separated on a 2% agarose gel, transferred to zetaprobe membrane and hybridized to ^{32}P-labeled internal oligonucleotide (primer 7 in Figure 4).

PCR
All PCRs were performed as described (Mahalingam *et al*, 1991), with minor modifications. Unless specified, all PCRs were cycled with denaturation

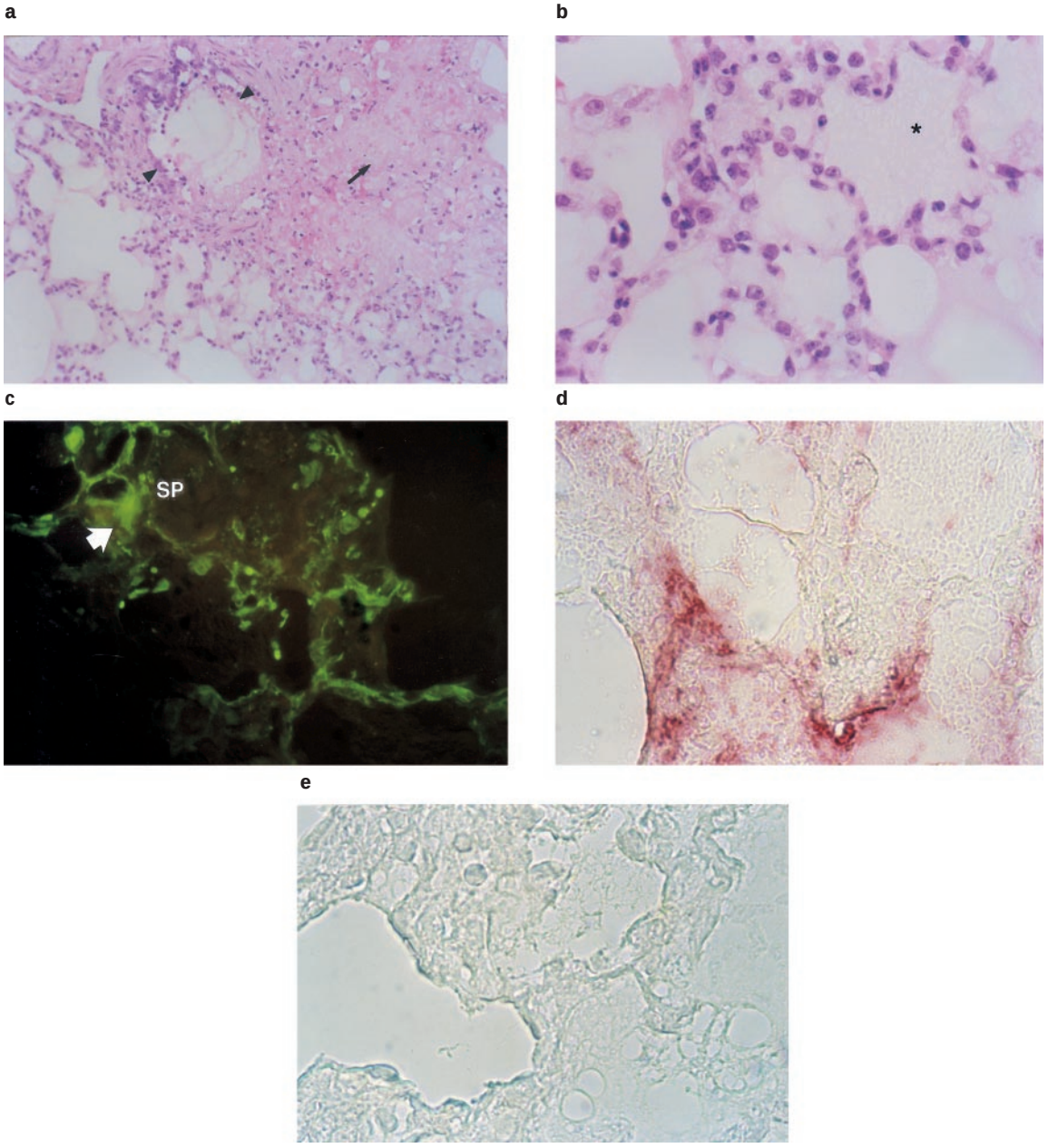

Figure 6 Lung from a 3-month-old monkey 10 days after infection with SVV–GFP. (**a**) Low power photomicrograph of the necrotizing pneumonitis (arrowheads) destruction of alveolar walls with proteinaceous and hemorrhagic exudate (arrow). Hematoxylin and eosin. ×175. (**b**) High power photomicrograph illustrates the intense mononuclear cell infiltration of aleveolar septae, as well as faintly eosinophilic alveolar proteinaceous exudate (asterisk) hematoxylin and eosin. ×525. (**c**) Green fluorescence under 490 nm ultraviolet light (white arrow) in an area of lung with intact alveoli indicates productive SVV–GFP infection. Alveolar space (SP). (**d**) SVV-specific antigen was detected in a section of the lung using rabbit anti-svv-antiserum and (**e**) no signal was seen using normal rabbit serum. ×216. These results (**a–e**) were all from sections that were within 50 microns of each other.

Table 1 Sequences of primers used for PCR

Primer	*Sequence (5′–3′)*
1	TGTCTGCTTAGGAGATTTTGGC
2	TAAAAAACGTCCTCGGATAGATGCATC
3	CCCGGGAGTGATAAGCGTT
4	CCCGGGGAATATACCGTAAC
5	GATATACCGGACCCATATCCCAACCC
6	GACGGCAGAACAAAACAAAATCCA
7	CAACCGGGCTTCTGTTTTATCTTCAA

for 1 min at 94°C, annealing for 2 min at 55°C, and elongation for 3 min at 72°C for a total of 34 cycles and a final cycle with denaturation, annealing and elongation times of 1, 2 and 7 min respectively. The sequences of the primers used are given in Table 1.

Western blot analysis of proteins from virus-infected cells

We scraped 90 mm tissue culture Petri dishes containing 10^6 uninfected or SVV-infected cells into 5 ml of DMEM, washed once in phosphate-buffered saline, and resuspended in 300 μl of lysis buffer (2% SDS, 0.34% NP-40). We passed the lysate through a Qia-shredder cartridge (Qiagen Inc., Chatsworth, CA) to reduce the viscosity, and determined the protein concentration in the eluate using a BCA protein assay system (Pierce Inc., Rockford, IL). We loaded 50 μg of the protein extracts onto an SDS-10%PAGE gel, prepared a Western blot as described (Towbin *et al*, 1979) and added rabbit anti-GFP antibody (Clontech Inc.) followed by alkaline phosphatase-conjugated goat anti-rabbit IgG, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for detection as described (Sambrook *et al*, 1989).

Inoculation of monkey with SVV expressing GFP and tissue collection

We infected a 3-month-old SVV-seronegative African green monkey intratracheally with 10^4 plaque forming units of SVV–GFP, and sacrificed the monkey when viremia appeared seven days later. There was no clinical evidence of varicella. The lungs were fixed in 4% paraformaldehyde, and 5 μm sections were stained with Harris hematoxylin and eosin and observed by light microscopy. Adjacent unstained sections were observed with an Orthoplan Universal large-field Leitz fluorescence microscope using a KP490 filter, 2× interference blue excitation filter and a K530 suppression filter.

Preparation of rabbit anti-SVV antiserum

SVV nucleocapsid preparations were resuspended in PBS and mixed with Freund's complete adjuvant for subcutaneous inoculation into rabbits. Rabbits were boosted once a month for 5 months with a mixture of SVV nucleocapsids and Freund's incomplete ajuvant. A 1:10 dilution of the rabbit antisera was adsorbed with uninfected BSC-1 cells at 37°C for 1 h and at 4°C for 16 h. The polyclonal sera was then adsorbed with normal monkey liver powder for 30 min and again for 20 h at 4°C. The antisera thus obtained was found to react specifically with SVV-infected cells but not with uninfected BSC-1 cells (data not shown).

Immunohistochemistry

Sections of monkey lung were analyzed using rabbit anti-SVV-antiserum as described (Mahalingam *et al*, 1996).

Acknowledgements

We thank Dr Roderic Smith for helpful discussion, Lisa Schneck and Mary Devlin for editorial review, and Cathy Allen for preparing the manuscript. Our research was supported by Public Health Service Grants AG 06127 and NS 32623. Tiffany White is the recipient of NIH Training Grant NS07321 in Neurovirology-Molecular Biology.

References

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994). Green fluorescent protein as a marker for gene expression. *Science* **263:** 802–805.

Clarke P, Rabkin SD, Inman MV, Cohrs R, Mahalingam R, Wellish M, Gilden DH (1992). Molecular analysis of simian varicella virus DNA. *Virology* **190:** 597–605.

Dueland AN, Martin JR, Devlin ME, Wellish M, Mahalingam R, Cohrs R, Soike KF, Gilden DH (1992). Acute simian varicella infection: Clinical, laboratory, pathological and virological features. *Lab Invest* **66:** 762–773.

Felsenfeld AD, Schmidt NJ (1977). Antigenic relationships among several simian varicella-like viruses and varicella-zoster virus. *Infect Immun* **15:** 807–812.

Gilden DH, Shtram Y, Friedmann A, Wellish M, Devlin M, Cohen A, Fraser N, Becker Y (1982). Extraction of cell-associated varicella-zoster virus DNA with triton X-100-NaCl. *J Virol Methods* **4:** 263–275.

Gray WL, Gusick NJ, Ek-Kommonen C, Kempson SE, Fletcher TM, III (1995). The inverted repeat regions of the simian varicella virus and varicella-zoster virus genomes have a similar genetic organization. *Virus Res* **39:** 181–193.

Mahalingam R, Smith D, Wellish M, Wolf W, Dueland AN, Cohrs R, Soike KF, Gilden DH (1991). Simian varicella virus DNA in dorsal root ganglia. *Proc Natl Acad Sci USA* **88:** 2750–2752.

Mahalingam R, Wellish M, Cohrs R, Debrus S, Piette J, Rentier B, Gilden DH (1996). Expression of protein encoded by varicella zoster virus open reading frame 63 in latently infected human ganglionic neurons. *Proc Natl Acad Sci USA* **93:** 2122–2124.

Padovan D, Canterell CA (1986). Varicella-like herpesvirus infections of nonhuman primates. *Lab Anim Sci* **36:** 7–13.

Sambrook J, Fritsch EF, Maniatis T (1989). *Molecular cloning: a laboratory manual,* Second edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 18.74.

Towbin H, Staehelin T, Gordon J (1979). Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:** 4350–4354.

Zolotukhin S, Potter M, Hauswirth WW, Guy J, Muzyczka N (1996). A 'humanized' green fluorescent protein cDNA adapted for high-level expression in mammalian cells. *J Virol* **70:** 4646–4654.