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Herpes Simplex Virus Type 1 Mutant Strain *in*1814 Establishes a Unique, Slowly Progressing Infection in SCID Mice

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Ocular infection of immunocompetent (BALB/c) mice with wild-type herpes simplex virus type 1 (HSV-1) 17$^+$ may lead to acute fatal encephalitis; however, in surviving animals, a latent (nonproductive) infection of the nervous system is established. In contrast, 17$^+$ infection invariably kills mice with severe combined immunodeficiency (SCID mice) within 2 weeks. Ocular infection of immunocompetent mice with a mutant HSV-1 strain, *in*1814, which does not produce a functional α-transinducing protein, results in no detectable viral replication in the nervous system during the time corresponding to the acute phase of infection, no mortality, and the establishment of latency. In SCID mice, however, the *in*1814 virus establishes a unique, slowly progressing infection. In studying the courses of *in*1814 infection in SCID and BALB/c mice, we found that although intact B- and/or T-lymphocytic functions were required for the control of viral replication in the nervous system, some of the infected neurons of SCID mice seemed to be able to restrict *in*1814 replication and harbor the virus in a latent state.

The failure of a host to eliminate a virus early after infection leads to death or the establishment of a persistent infection that may or may not be associated with disease and a shortened life span. Persistent viral infections have traditionally been divided into two categories (reviewed in references 2 and 30): (i) chronic productive infections in which infectious virus can be recovered at all times and (ii) latent infections in which the viral genome is present but infectious virus is not produced except during intermittent episodes of reactivation. Although recent advances in many areas of virology have shown that the division between these two categories is not always clear-cut, the lifelong persistence of herpes simplex virus type 1 (HSV-1) in humans and laboratory animals has not been challenged as the paradigm of latent, nonproductive infection (2, 15).

Most of our present knowledge concerning the pathogenesis of HSV-1 persistence comes from studies done with laboratory animals (for reviews, see references 13, 16, 47, and 48). The mouse eye–trigeminal-ganglion HSV-1 latency model (21, 22) has proved exceptionally useful in these studies. In this model, following ocular infection, HSV-1 first replicates in the eyes and then travels to the trigeminal ganglia by fast axonal transport. It replicates there, as it does later in the central nervous system. Some animals may die during the acute phase of infection of encephalitis; however, in surviving animals, viral replication subsides by approximately 2 weeks postinfection (p.i.). By 4 weeks p.i., HSV-1 can be found only in the nervous system (mainly in the trigeminal ganglia) in a nonreplicative, latent form (13, 22, 48). Virus can be reactivated from latency by different methods, including immunosuppression. Although spontaneous reactivation does occur in guinea pig and rabbit models, it is extremely infrequent in mice if it occurs at all (17, 22, 31, 39, 52, 54).

It is clear that HSV-1 persistence is controlled by special immunological, cellular (neuronal), and viral factors. The crucial role of acquired immunity in the regulation of HSV-1 persistence has been shown by numerous studies with laboratory animals (reviewed in references 28 and 37). It has been demonstrated that acquired immunity controls HSV-1 replication both in peripheral tissues and in the nervous system (8, 19, 26, 31, 40). HSV-1 persistence is not established in immunodeficient mice (nude and with severe combined immunodeficiency [SCID] mice), as these die of encephalitis shortly after infection (25–27, 31). In immunocompetent mice latently infected with HSV-1, the immune system has a role in keeping the animals free of infectious virus; hence, as previously mentioned, immunosuppressive treatment can result in viral recrudescence (26, 31).

It is presently unclear what role, if any, the immune system plays in the establishment and maintenance of latency in the neurons. It is thought that HSV-1 can hide in some of the infected neurons and avoid elimination by the immune system. Neurons fail to express major histocompatibility complex determinants and thus provide viruses an ideal place to hide from the immune system (reviewed in reference 30). Following infection, HSV-1 is thought to enter (and later maintain) a nonreplicative, latent state in some neurons immediately after it reaches the nervous system (2, 45, 50). During latency, HSV-1 replication is probably blocked at the level of viral immediate-early (IE) gene expression (the first genes expressed during the replication cycle in tissue culture cells) (36, 43, 49, 51). The special ability of neurons to restrict HSV-1 replication during latency has been ascribed to their differentiated state, in which transcription factors required for viral IE gene expression are missing or repressor factors are present (20, 38, 50). It is, however, possible that it is not only the special physiology of neurons that leads to the restriction of viral gene expression during the establishment and maintenance of HSV-1 latency. Experimental evidence suggests that antibody can bind to HSV-1-infected neuronal cells and suppress intracellular virus replication (29). Furthermore, CD8$^+$ cells may promote the termination of viral gene expression in neurons without inducing cytolysis (41). It is clear that further studies

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are required to define the exact roles of neuronal and immune factors in the regulation of HSV-1 latency in neurons.

One of the viral factors thought to be important regulators of HSV-1 replication and latency is the viral α-transinducing protein Vmw65 (1, 33, 45). This protein is encoded by a gene expressed late during the replication cycle and is incorporated in the tegument part of the virion (42). The function of the Vmw65 protein is to transactivate viral IE genes after infection, and it is also important for viral assembly (36). It has been hypothesized that being a tegument protein, Vmw65 may get left behind during the long axonal travel of the viral material to the soma of sensory neurons, and thus its function would be missed there, resulting in inefficient IE gene expression and latency (36, 45).

A mutant HSV-1 virus, *in*1814 (1), which contains an insertion into its Vmw65 gene that affects the transinducing activity of the gene product, was found not to replicate in the nervous systems of mice but to establish latency there following ocular infection (45). In recent studies, however, we have shown that although *in*1814 establishes latency in most infected neurons of the trigeminal ganglia immediately after reaching these cells, some signs of replication (namely, expression of acute-phase HSV-1 genes) still occur in a small portion of the infected neurons shortly after infection (51). This observation raised the possibility that in addition to the impaired ability of *in*1814 to replicate in neurons, an immune response might have caused the inability to detect infectious particles in the nervous system during the acute phase of *in*1814 infection.

In the present study, we infected immunodeficient SCID mice (deficient in both T- and B-lymphocytic functions [3]) with HSV-1 *in*1814 to examine how the lack of a specific immune response to the infecting virus would affect *in*1814-neuronal interactions. More specifically, we designed experiments to test whether it was possible to establish HSV-1 latency in animals deficient in their T- and B-lymphocytic functions. We found that *in*1814 infection of SCID mice leads to a unique, slowly progressing infection characterized by the detectability of infectious virus and replicative viral gene expression for 4 to 6 weeks p.i., gradually increasing virus-specific DNA levels in the nervous system as a function of time p.i., and a mean survival time until death of 44.6 ± 26 days p.i. This finding shows that B- and/or T-cell-mediated immune responses are required to control HSV-1 replication in the nervous system even if the viral strain used for infection lacks neurovirulence in the immunocompetent host. However, most neurons of SCID mice seem to be able to restrict HSV-1 *in*1814 replication, suggesting that no B- or T-lymphocytic functions are required for the establishment and maintenance of HSV-1 latency in some neurons in vivo.

MATERIALS AND METHODS

Virus stocks. To produce virus stocks, subconfluent monolayers of baby hamster kidney 21 clone 13 (BHK) cells were infected with HSV-1 17^+ (5) or insertion mutant *in*1814 (1). The tissues were titrated on BHK cells. The titers of viral stocks used were 4×10^8 PFU/ml for HSV-1 17^+ and 4×10^6 PFU/ml for *in*1814.

Animals. Female BALB/c mice (4 to 6 weeks old) were obtained from the Jackson Laboratory. Immunodeficient male and female SCID (CB-17 *scid/scid*) mice, originally obtained from M. Bosma (Fox Chase Cancer Center, Philadelphia, Pa.), were bred and maintained in a pathogen-free environment at The Wistar Institute animal facility. Serum immunoglobulin M titers of the mice were routinely tested by a direct enzyme-linked immunosorbent assay when the mice were 6 to 7 weeks old, and only non-immunoglobulin M-producing mice were used in our studies.

Infection of mice. BALB/c (5- to 10-week-old) and SCID (7- to 12-week-old) mice were inoculated after corneal scarification with 2×10^4 PFU of HSV-1 17^+ or *in*1814 or with serum-free Dulbecco modified Eagle medium (mock infection). At selected times (4, 9, 15, or 26 to 42 days p.i.), mice were sacrificed by cervical dislocation, and the eyes, trigeminal ganglia, and brain stems were removed aseptically and processed for plaque assay and RNA or DNA studies. For mortality studies, groups of HSV-1-infected or mock-infected animals were observed for 4 months.

Detection and identification of infectious HSV-1 in the eyes, trigeminal ganglia, and brain stems of infected animals. 17^+, *in*1814-, or mock-infected BALB/c and SCID mice were sacrificed 4 to 6 weeks p.i. The eyes, trigeminal ganglia, and brain stems from each animal were homogenized separately in 2 ml of medium without serum. Aliquots (1 ml each) of the homogenates were inoculated onto monolayers of BHK cells grown in six-well tissue culture plates. Virus grown in plates inoculated with homogenates of tissues from *in*1814-infected mice was used to infect CV-1 monolayers. Nucleocapsid-associated DNA was extracted from cytoplasmic fractions of infected cell cultures, restricted with *Bam*HI, electrophoresed in a 0.8% agarose gel, transferred onto nitrocellulose, and hybridized with a ^{32}P-labeled nick-translated HSV-1 restriction fragment *Bam*HI-F probe (46).

In situ hybridization for HSV-1-specific gene expression. In situ hybridization was performed as previously described (10). Sections of eyes, trigeminal ganglia, and brain stems from eight SCID mice sacrificed 4 to 6 weeks p.i. were mounted on slides and studied individually for each animal. Tissues derived from BALB/c mice 4 to 6 weeks p.i. were pooled. For each combination of probe, tissue, and animal, at least three sections were studied. The positive cells in each section were counted, and the mean number of positive cells per section was calculated.

Probes for in situ hybridization. Plasmid pBR322, containing *Eco*RI-*Bam*HI fragment I/I (KOS; glycoprotein C [gC] probe) was obtained from E. K. Wagner and R. J. Frink, University of California, Irvine (14). Plasmid pRB113 (*Bam*HI-Y; ICP4 probe) and plasmid pRB112, containing *Bam*HI-B, were obtained from B. Roizman, University of Chicago (33). The latency-associated transcript (LAT) probe *Bst*EII-*Bst*EII was isolated from the *Bam*HI B fragment. Plasmid pBR322, containing the *Bam*HI fragment a' (KOS) (7) and used for a VP5 probe, was obtained from E. K. Wagner and R. H. Costa (University of California, Irvine). DNA inserts were gel purified and nick translated as described previously (10). The specific activities of the nick-translated ^{35}S-labeled probes were 1×10^8 to 2×10^8 cpm/μg.

RNA extraction and Northern (RNA) blot analysis. Trigeminal ganglia and brain stems were quick frozen in liquid nitrogen and stored at −70°C. Total RNA was prepared from pooled trigeminal ganglia (two to five animals) and from single brain stems by using the guanidium thiocyanate extraction-cesium chloride centrifugation procedure of Chirgwin et al. (6). Northern analysis was performed as previously described (43) with a few modifications. Briefly, 10 μg of glyoxal-treated RNA per lane was electrophoresed, vacuum blotted (LKB) to a nylon membrane (GeneScreen Plus; NEN), and baked at 80°C for 2 h. Hybridization was carried out overnight with heat-denatured ^{32}P-labeled nick-trans-

TABLE 1. Mortality of BALB/c and SCID mice after ocular infection with HSV-1 17^+ or *in*1814

Mouse type	No. of mice	Infection (HSV-1 strain or mock)	MOI (PFU/eye)	Time of death (days p.i.)	Length of observation (days)	Mean time of deaths (days)	Mortality (%)
BALB/c	5	Mock			120		0
BALB/c	10	17^+	2×10^4		120		0
BALB/c	10	*in*1814	2×10^4		120		0
SCID	5	Mock			120		0
SCID	5	17^+	2×10^4	7, 7, 8, 8, 9	9	7.8 ± 0.8	100
SCID	5	17^+	2×10^2	9, 11, 11, 13, 14	14	11.6 ± 1.9	100
SCID	10	*in*1814	2×10^4	17, 23, 28, 40, 51, 61, 92	120	44.6 ± 26	70

lated DNA probes (43). Probes used were *Eco*RI-*Bam*HI fragment I/I (as described above; gC probe) and fragment *Pst*I-*Bam*HI isolated from plasmid pRB112 (33; LAT-ICP0 probe). Filters underwent a series of two 25-min washes at 65°C in 1×, 0.5×, and 0.1× SSPE (1× SSPE is 180 mM NaCl, 10 mM monobasic sodium phosphate [pH 7.7], and 1 mM EDTA) with 1% sodium dodecyl sulfate. For autoradiography, filters were exposed to XAR-5 film (Eastman Kodak) for 2 days.

Extraction and quantification of viral DNA from trigeminal ganglia. DNA was extracted as described previously (34). DNA (5 μg) from each sample (three pairs of trigeminal ganglia) was spot blotted onto nitrocellulose filters with a manifold apparatus (Schleicher & Schuell) as previously described (18, 24). The nitrocellulose filters were baked for 2 h at 80°C, hybridized with a nick-translated ^{32}P-labeled HSV-1 (F) virion DNA probe, washed, and processed for autoradiography as previously described (11). The radioactivity counts of the samples was determined by liquid scintillation counting in Econoflour. Quantification of HSV-1 genomes per cell was by comparison with HSV-1 standards as previously described (44).

Southern blot analysis of viral DNA from trigeminal ganglia and brain stems of *in*1814-infected BALB/c and SCID mice. DNA extracted from trigeminal ganglia and brain stems was digested with *Bam*HI, electrophoresed in agarose gels, transferred to nitrocellulose filters, and hybridized with a nick-translated ^{32}P-labeled DNA probe as described previously (34).

RESULTS

Mortality of immunocompetent and immunodeficient mice after ocular infection with wild-type (17^+) or mutant (*in*1814) HSV-1 strains. Ocular infection of BALB/c mice with 2×10^4 PFU (a relatively low dose) of wild-type HSV-1 17^+ or *in*1814 per eye was not lethal to any of the animals (0% mortality; Table 1; 45). However, infection of SCID mice with even 100-fold fewer strain 17^+ infectious particles per eye killed all mice within 14 days (Table 1). Ocular infection of SCID mice with 2×10^4 PFU of *in*1814 per eye, however, killed 70% of the animals (observation time, 120 days), with a mean survival time until death of 44.6 ± 26 days p.i. None of the mock-infected BALB/c or SCID mice died during the 4-month observation period.

Isolation of infectious HSV-1 from the eyes, trigeminal ganglia, and brain stems of SCID mice 4 to 6 weeks after infection with *in*1814. In accordance with previous studies by this and other laboratories, infectious HSV-1 was never detected in the eyes, trigeminal ganglia, or brain stems of BALB/c mice at 28 days after infection with 17^+ or *in*1814 (Table 2). In fact, infectious *in*1814 was not recovered from the trigeminal ganglia or brain stems of BALB/c mice at any time (45). In contrast, as shown in Table 2, *in*1814 could be isolated from the eyes and nervous systems of some SCID mice (three of six) 4 to 6 weeks p.i. It should be noted that the plaque assay for infectious HSV-1 is less sensitive in detecting *in*1814 than 17^+, probably because of the impaired ability of *in*1814 to replicate in tissue culture cells at low multiplicities of infection (MOIs). Thus, it is possible that infectious particles were present in some of the tested tissues at levels below the sensitivity of our assay. Southern blot analysis confirmed that the isolated virus was *in*1814 (Fig. 1). The *Bam*HI restriction pattern of the isolated virus was the same as that of the input *in*1814 stock and differed from the restriction pattern of 17^+ DNA only in the *Bam*HI-F region (Fig. 1), which suggests that isolated virus and input *in*1814 differed from 17^+ only in the structure of the Vmw65 gene (data not shown).

Viral gene expression in eyes, trigeminal ganglia, and brain stems of BALB/c and SCID mice infected with HSV-1 17^+ and *in*1814 as detected by in situ hybridization. Numerous studies

TABLE 2. Presence of infectious virus and virus-specific mRNAs of different kinetic classes in eyes, trigeminal ganglia, and brain stems of BALB/c and SCID mice 4 to 6 weeks after infection with HSV-1 17^+ or *in*1814[a]

Mouse type	HSV-1 infecting strain[b]	No. of mice positive/total no. tested by:														
		Plaque assay			In situ hybridization											
					LAT			ICP4 (IE)			VP5 (early)			gC (late)		
		Eyes	TG	BST	Eyes	TG	BST	Eyes	TG	BST	Eyes	TG	BST	Eyes	TG	BST
BALB/c	17^+	0/9	0/9	0/9	0/4	9/9	3/4	0/4	0/9	0/4	ND	0/9	ND	0/4	0/9	0/4
SCID	17^+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BALB/c	*in*1814	0/9	0/9	0/9	0/4	9/9	2/4	0/4	0/9	0/4	ND	0/9	ND	0/4	0/9	0/4
SCID	*in*1814	3/6	3/6	2/6	6/8	8/8	8/8	6/8	7/8	7/8	6/8	7/8	7/8	6/8	8/8	7/8

[a] TG, trigeminal ganglia; BST, brain stems; NA, no mice alive by 4 to 6 weeks p.i.; ND, not done.
[b] MOI, 2×10^4 to 1×10^5 PFU.

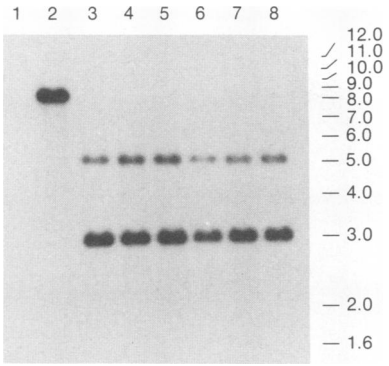


FIG. 1. (A) Southern blot analysis for strain identification of HSV-1 isolated from *in*1814-infected SCID mice 4 to 6 weeks p.i. DNAs from mock-infected CV-1 cells and from cells infected with isolated virus, 17^+, or *in*1814 were restricted with *Bam*HI and probed with ^{32}P-labeled nick-translated HSV-1 restriction fragment *Bam*HI F. Lanes: 1, DNA from mock-infected CV-1 cells; 2 and 3, DNAs from CV-1 cells infected with HSV-1 17^+ or *in*1814, respectively; 4 to 8, DNAs from CV-1 cells infected with virus isolated from the trigeminal ganglia (lanes 4 to 6), eyes (lane 7), and brain stems (lane 8) of individual mice 4 to 6 weeks after infection with HSV-1 *in*1814. A single 8-kb HSV-1 band was present in DNA derived from 17^+-infected CV-1 cells. Two bands, 5 and 3 kb in size, were present in DNA derived from CV-1 cells which were infected with *in*1814 or virus isolated from the eyes, trigeminal ganglia, or brain stems of SCID mice 4 to 6 weeks after infection with *in*1814. This band pattern is characteristic of *in*1814, which carries an additional *Bam*HI restriction site in the *Bam*HI-F region compared with strain 17^+ (1).

have shown that by 4 weeks after ocular infection of immunocompetent mice, HSV-1 is present in the nervous system in a latent, nonreplicative state. This state is characterized by a restricted pattern of viral gene expression: only the LATs are transcribed from the viral genome (9, 10, 43, 49). In agreement with that, we could not detect expression from ICP4, VP5, and gC viral genes, which belong to the IE, early-late, and late kinetic classes of replicative HSV-1 genes, respectively, in the trigeminal ganglia of 17^+- or *in*1814-infected BALB/c mice 4 to 6 weeks p.i. LAT mRNA, however, was readily detected in the trigeminal ganglia and less frequently in the brain stems of 17^+- and *in*1814-infected animals (Fig. 2, Tables 2 and 3).

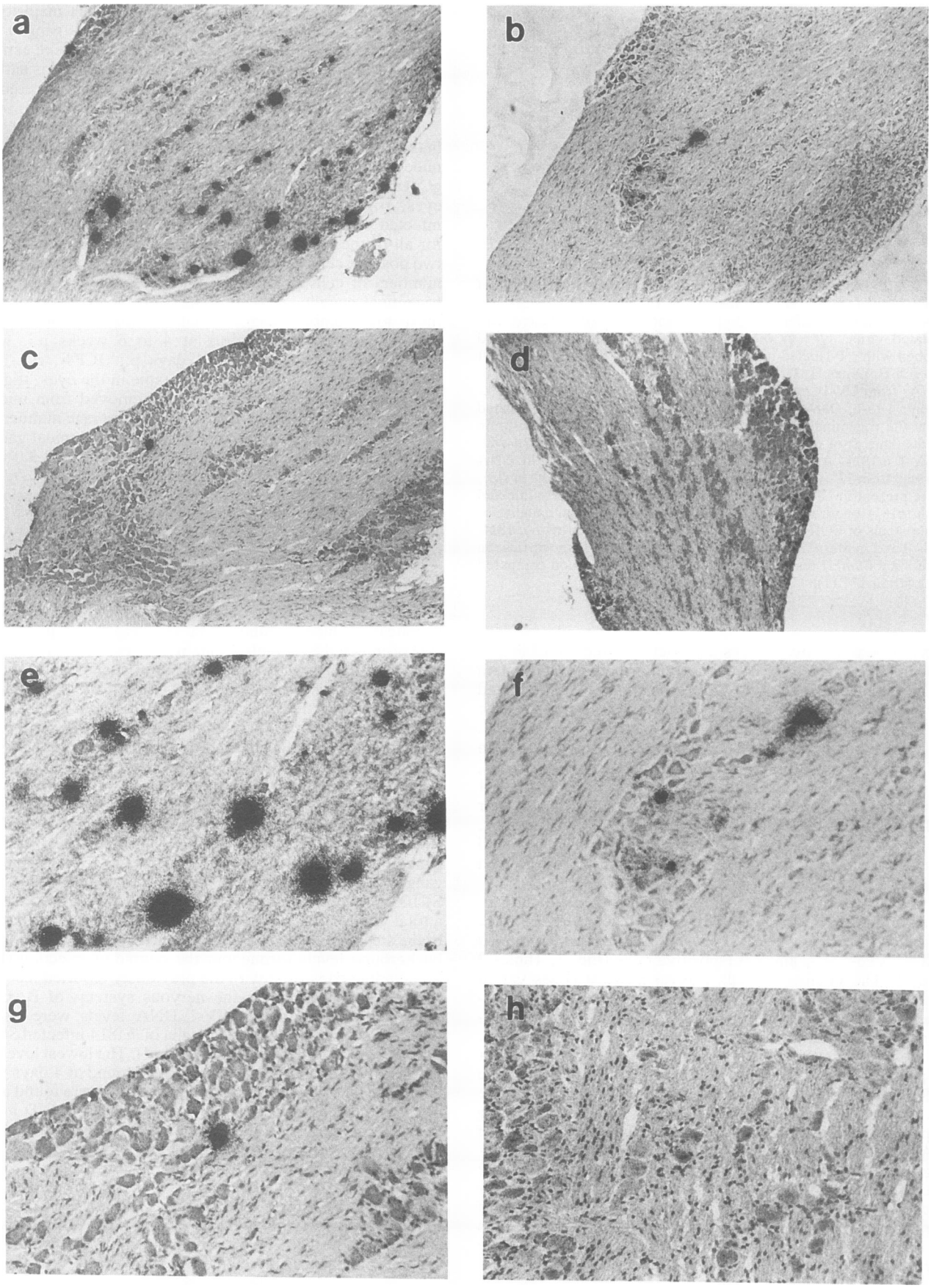
In sharp contrast, most tissue sections of trigeminal ganglia, brain stems, and eyes derived from eight SCID mice sacrificed 4 to 6 weeks after *in*1814 infection contained ICP4-, VP5-, gC-, and LAT-positive cells (Fig. 2, Tables 2 and 3). The number of cells expressing viral genes varied from animal to animal (Table 3), possibly because of experimental variation in the infection procedure and/or differences in the extent of immunodeficiency of the animals (4). In the trigeminal ganglia and brain stems, the largest number of positive cells was usually seen with the LAT probe, while mRNAs for ICP4 and gC were detected in fewer cells (Table 3). In contrast, in the eyes, the number of cells expressing replicative genes was usually higher than the number of LAT-expressing cells. Careful analysis revealed the following pattern of viral gene expression in the trigeminal ganglia: the number of LAT-positive neurons was significantly higher than the number of neurons positive for the expression of replicative genes. In contrast, non-neuronal (satellite) cells in the trigeminal ganglia were about as frequently positive for mRNAs of replicative genes as for the LATs.

This numerical analysis suggests that in the majority of neurons containing viral RNA in the trigeminal ganglia and brain stems of *in*1814-infected SCID mice, viral gene expression was limited to the LATs at 4 to 6 weeks p.i. This pattern of limited viral gene expression is generally considered an indication of latent HSV-1 infection.

The patterns of viral gene expression in the eyes, trigeminal ganglia, and brain stems of 17^+- or *in*1814-infected SCID mice were also determined at 4 days p.i. At that time, ICP4-, gC-, and LAT-positive cells were readily detectable in the eyes, trigeminal ganglia, and brain stem tissues of mice infected with *in*1814; however, the numbers of cells positive for all of the studied mRNAs were low (fewer than one or two positive cells per section). When compared with the high numbers of cells positive for viral transcripts in the eyes, trigeminal ganglia, and brain stems by 4 to 6 weeks p.i., these low numbers suggest that the majority of nervous system cells harboring virus at 4 to 6 weeks p.i. were probably infected later than 4 days p.i. ICP4-, gC-, and LAT-positive cells were also detectable in the eyes, trigeminal ganglia, and brain stem tissues removed from mice 4 days after infection with strain 17^+. The average numbers of neurons positive for these transcripts per trigeminal ganglionic section were 8.5 with ICP4, 7.0 with gC, and 3.0 with LAT.

Northern blot hybridization study of viral gene expression in trigeminal ganglia and brain stems of *in*1814-infected BALB/c and SCID mice 4 weeks p.i. Two LATs (2 and 1.5 kb) were detected in the trigeminal ganglia of *in*1814-infected BALB/c and SCID mice, and the more-abundant 2-kb LAT (43) was also detected as a faint band in brain stem extracts from two of four *in*1814-infected SCID mice (Fig. 3). The LATs were significantly more abundant in the trigeminal ganglia of *in*1814-infected SCID mice than in BALB/c mice. The LATs are transcribed from the DNA strand opposite that encoding ICP0, an IE transcript (43, 49). Although the *Pst*I-*Bam*HI probe used for the detection of LAT expression had the potential to detect ICP0 transcription, only LAT expression was above detection levels in the tissues studied. By the Northern blot technique, we could not detect gC expression in the brain stems of *in*1814-infected SCID mice 4 weeks p.i.; however, we detected low-level gC expression in the trigeminal ganglia (data not shown).

Detection and quantification of HSV-1 DNAs in trigeminal ganglia and brain stems. DNAs extracted from trigeminal ganglia and brain stems of *in*1814-infected BALB/c and SCID mice were quantified by spot blot hybridization using a total virion DNA probe. HSV-1 DNA levels in ganglia and brain stems from *in*1814-infected BALB/c mice were near background levels throughout the course of infection (Fig. 4), a finding consistent with the previously reported inability of *in*1814 to replicate in the nervous systems of BALB/c mice (45). In contrast, HSV-1 DNA levels were above background in the trigeminal ganglia of *in*1814-infected SCID mice at every studied time point (Fig. 4). The lowest levels of viral DNA (0.06 genome per cell) were found at 4 days p.i., and the highest levels (0.35 genome per cell) were found at 28 days p.i. In the brain stems of *in*1814-infected SCID mice, HSV-1-specific DNA levels were near background at 4, 7, and 14 days p.i. but reached a higher level (0.07 copy per cell) by 28 days p.i. (Fig. 4). These findings suggest active HSV-1 replication in the nervous systems of SCID mice throughout the studied period p.i. and demonstrate the difference between the SCID and BALB/c HSV-1 models, as previous studies (34, 35, 51) have shown that in the nervous systems of immunocompetent mice, HSV-1 DNA reaches its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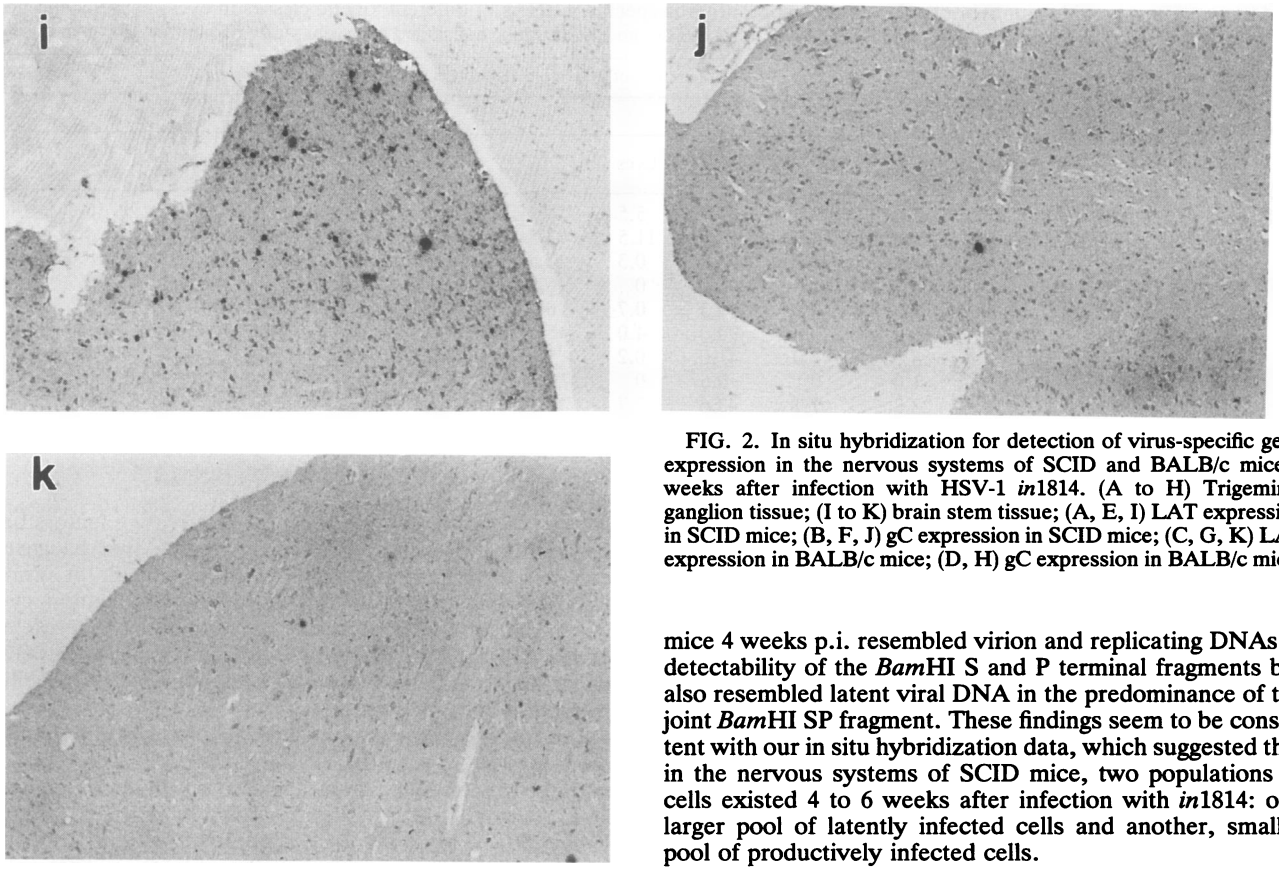

FIG. 2. In situ hybridization for detection of virus-specific gene expression in the nervous systems of SCID and BALB/c mice 4 weeks after infection with HSV-1 *in*1814. (A to H) Trigeminal ganglion tissue; (I to K) brain stem tissue; (A, E, I) LAT expression in SCID mice; (B, F, J) gC expression in SCID mice; (C, G, K) LAT expression in BALB/c mice; (D, H) gC expression in BALB/c mice.

highest levels during the first 2 weeks of infection and remains at low and stable levels later. At 28 days p.i. (i.e., during the traditional latent phase of infection), HSV-1 DNA levels in the trigeminal ganglia of SCID mice (0.35 copy per cell) were higher than published levels of wild-type HSV-1 DNA in latently infected immunocompetent mice (0.1 ± 0.09 copy per cell) (12, 34, 44, 51) and were significantly higher than HSV-1 *in*1814 DNA levels detected in latently infected BALB/c mice (<0.02 copy per cell) previously (51) and in this study.

Physical state of HSV-1 DNAs in trigeminal ganglia and brain stems of *in*1814-infected mice. Previous studies have shown that virion, replicating, and latent viral DNAs are physically different. Virion DNA is linear. Following infection, it circularizes (32), and replication is initiated from the circular form, resulting in the synthesis of long linear concatemers. The transition from the linear to the circular or concatemeric state is marked by reduction of the terminal fragments (*Bam*HI-S and -P) in Southern blots of *Bam*HI-digested DNA (34). Latent HSV-1 DNA completely lacks terminal fragments (34, 35). Southern blot analysis of *Bam*HI-restricted DNAs derived from the trigeminal ganglia and brain stems of *in*1814-infected SCID mice 4 weeks p.i. revealed an almost complete absence of terminal fragments *Bam*HI S and P and a predominance of the junction *Bam*HI SP fragment (Fig. 5). (*Bam*HI-S and -P were visible as very weak bands on the autoradiographs; however, by densitometric scanning, we could not detect higher-than-background counts at the sites corresponding to the bands seen.) Thus, the revealed physical state of HSV-1 DNAs in the trigeminal ganglia and brain stems of *in*1814-infected SCID mice 4 weeks p.i. resembled virion and replicating DNAs in detectability of the *Bam*HI S and P terminal fragments but also resembled latent viral DNA in the predominance of the joint *Bam*HI SP fragment. These findings seem to be consistent with our in situ hybridization data, which suggested that in the nervous systems of SCID mice, two populations of cells existed 4 to 6 weeks after infection with *in*1814: one larger pool of latently infected cells and another, smaller pool of productively infected cells.

DISCUSSION

It is well known (25–27, 31) and we have shown again in this paper that an immunocompetent state is required for long-term survival of wild-type-HSV-1-infected hosts and thus also for the establishment of viral persistence. Ocular infection of immunocompetent (BALB/c) mice with a relatively low dose (2×10^4 PFU per eye) of wild-type HSV-1 17^+ was associated with no mortality, and we demonstrated that a latent (nonproductive) type of persistent HSV-1 infection was established in the nervous systems of these mice by 4 weeks p.i. Also, as previously shown (45), ocular infection of BALB/c mice with *in*1814, a virion-transactivating-factor mutant HSV-1 strain, was associated with no mortality but with the establishment of viral latency in the nervous system by 4 weeks p.i. In contrast, ocular infection of SCID mice with strain 17^+ killed all infected mice within 2 weeks.

SCID mice survived significantly longer following *in*1814 infection than following 17^+ infection. By isolating infectious virus, detecting replicative-viral-gene expression for 4 to 6 weeks p.i., and finding gradually increasing virus-specific DNA levels in the nervous systems of SCID mice as a function of time p.i., we demonstrated that *in*1814 established a unique type of persistence: a slowly progressing, productive infection.

HSV-1 is a lytic virus. Thus, in order to achieve long-term persistence in the infected host, it must avoid killing too many host cells (which would lead to the early death of the host) and must also avoid elimination by the host immune system (2). In immunocompetent animals, HSV-1 is thought to be able to achieve persistence by establishing a nonlytic (latent) type of interaction with some of the infected neu-

TABLE 3. Results of in situ hybridization for detection of virus-specific mRNAs of different kinetic classes in eyes, neurons and non-neuronal cells of trigeminal ganglia, and brain stems of BALB/C and SCID mice sacrificed 4-6 weeks after ocular *in* 1814 infection

Mouse type	Animal no.	Mean no. of positive cells/section for expression of:											
		LAT				ICP4				gC			
		Eyes	TG neurons	Other TG cells	Brain stems	Eyes	TG neurons	Other TG cells	Brain stems	Eyes	TG neurons	Other TG cells	Brain stems
SCID	1	1.2	73.5	23.5	69.0	5.5	14.3	19.6	4.2	5.0	12.5	15.5	3.0
	2	3.6	29.5	6.0	14.0	11.5	7.5	8.6	5.5	8.0	3.0	4.0	3.5
	3	0	42.5	11.5	98.0	0.3	17.5	17.6	9.5	0.3	21.0	18.0	11.0
	4	0	20.5	10.5	35.5	0	5.0	23.3	3.0	0	5.3	19.7	1.7
	5	0.5	0.5	0	1.3	0.7	0	1.0	0.2	0.7	0	0	0.5
	6	3.0	55.0	6.0	90.0	4.0	15.0	15.0	5.3	4.0	4.0	2.0	0.6
	7	0.5	8.0	4.0	41.0	0.2	4.0	4.0	1.6	1.0	4.0	3.0	0.7
	8	0	1.0	0	0.5	0	0	0	0	0	0	0.5	0
	Mean	1.1	28.8	7.7	43.6	2.7	7.9	11.1	3.6	2.3	6.2	7.8	2.6
BALB/c (n = 9)	Mean	0	1.0	0.1	0.5	0	0	0	0	0	0	0	0

rons. Following infection, cells in which viral replication occurs are lysed by the virus or eliminated by the immune system. Latently infected neurons are not eliminated, because neither replication nor elimination by the immune system occurs.

*in*1814 can achieve a novel type of viral persistence in SCID mice, probably for the following reasons. Immune surveillance does not occur in SCID mice, so viral replication cannot be controlled at the site of inoculation or in the nervous system. However, because of its defect in the Vmw65 transactivating function, *in*1814 may replicate inefficiently in vivo, as it does at low MOIs in vitro (1). Thus, it can take a long time until lethal damage to the host is achieved. In most cells infected by this virus, viral IE-gene expression may not reach the levels required for further progression of the replication program, thus resulting in viral latency (36, 51). However, viral replication still occurs in some of the infected cells. This may be due either to sufficient amounts of tegument proteins (including transactivating protein ICP4; 55) reaching the nucleus of the infected cell or to high levels of cellular transactivating factors being present in these cells. It is also possible that because of compromised virion assembly (36) or a lack of Vmw65 transactivation function late during the replication cycle, when the newly synthesized mutated Vmw65 protein is expressed, the replication of *in*1814 is hampered in cells in which an initial IE-gene transactivation (mediated by cellular or viral factors) occurs.

Our findings show that B- and/or T-lymphocytic functions are required to control HSV-1 infection in the nervous system, even if the viral strain used for infection seems to lack neurovirulence (1, 45) in immunocompetent animals. However, the majority of infected neurons in the trigeminal ganglia of SCID mice seem to be able to restrict *in*1814 replication and harbor the virus in a latent form. This was

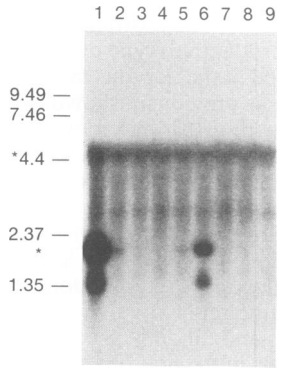

FIG. 3. Detection of HSV-1 LATs by Northern blot analysis in the trigeminal ganglia (TG) and brain stems (BS) of SCID and BALB/c mice 4 weeks after corneal infection with HSV-1 *in*1814. Lanes were loaded with 10 μg of RNA extracted as described in Materials and Methods. Lane 1, SCID TG; lanes 2 through 5, SCID BS; lane 6, BALB/c TG; lanes 7 and 8, BALB/c BS; lane 9, uninfected TG. The nick-translated ^{32}P-labeled probe was fragment *Pst*I-*Bam*HI, which has the potential to detect both LAT and ICP0 RNAs. The positions of RNA markers (in kilobases) are labeled on the left, and the positions of 28S and 18S rRNAs are marked with asterisks.

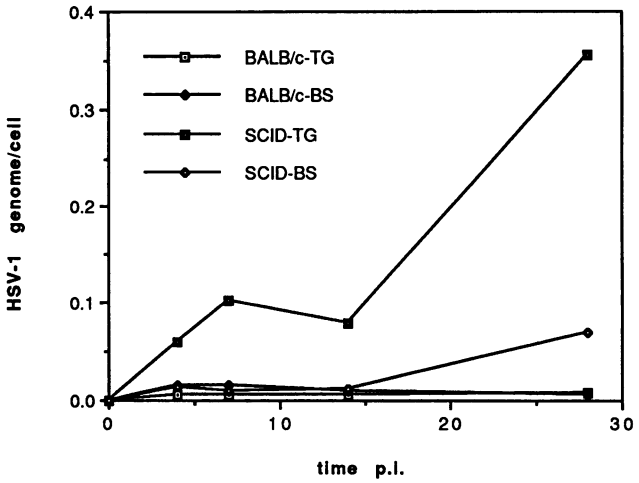

FIG. 4. Detection of HSV-1-specific DNAs in the trigeminal ganglia and brain stems of mice during the course of *in*1814 infection. DNA (5 μg) extracted from three pairs of trigeminal ganglia or individual brain stems of *in*1814-infected BALB/c or SCID mice was analyzed by dot blot hybridization with a total HSV-1 virion DNA probe. Spot blot radioactivity was determined by liquid scintillation counting in Econofluor. Quantification of HSV-1 genomes per cell was by comparison with HSV-1 standards. Data shown represent the mean levels of HSV-1-specific DNA for each time point as calculated from three to seven individual brain stems and one or two pools of three pairs of trigeminal ganglia. TG, trigeminal ganglia; BS, brain stems.

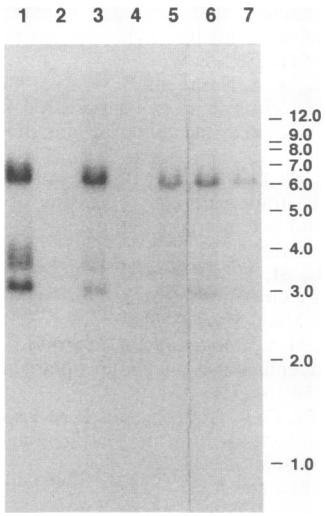

FIG. 5. Southern blot hybridization of nick-translated ^{32}P-labeled *Bam*HI SP fragment DNA to *Bam*HI digests of trigeminal ganglion and brain stem DNAs taken from *in*1814-infected SCID and BALB/c mice 4 weeks p.i. DNA was extracted as described in Materials and Methods. Lane 1, 1.0 ng of HSV-1 17$^+$ DNA and 5 μg of trigeminal ganglion DNA from uninfected mice; lane 2, 5 μg of trigeminal ganglion DNA from uninfected mice; lane 3, 5 μg of DNA from three pairs of trigeminal ganglia of mice 4 days after infection with strain 17$^+$ (MOI = 3.3×10^6 PFU per eye); lane 4, 5 μg of DNA from three pairs of trigeminal ganglia of *in*1814-infected BALB/c mice sacrificed 4 weeks p.i.; lane 5, 5 μg of DNA from three pairs of trigeminal ganglia of *in*1814-infected SCID mice 4 weeks p.i.; lanes 6 and 7, 5 μg of DNA from the brain stems of two SCID mice sacrificed 4 weeks after infection with *in*1814. Numbers on the right indicate positions of DNA markers (in kilobases).

suggested by the Southern blot analysis of the structure of *in*1814 DNA at 4 to 6 weeks p.i., which revealed that the majority of that DNA was present in an endless form, a characteristic feature of latent HSV-1 DNA (34, 35). Our Northern blot hybridization studies showed massive expression of the LATs versus hardly detectable gC and nondetectable ICP0 expression in the trigeminal ganglia of *in*1814-infected SCID mice. Similarly, in situ hybridization data (Fig. 2, Tables 2 and 3) suggested that the number of LAT-positive neurons in the trigeminal ganglia of SCID mice was on the average four times higher than the number of neurons positive for replicating genes at 4 to 6 weeks p.i. (Table 3). Thus, it seems likely that viral gene expression in most neurons was limited to the LATs. Although LAT transcription occurs in both productively and latently infected cells, HSV-1 gene expression limited to the LATs is generally considered an indication of a latent infection. It should be noted that the specific activities of the probes used to detect expression of the LATs and those of replicative genes were not different; thus, it seems likely that our in situ hybridization experiments were equally sensitive for the detection of the different HSV-1 mRNAs studied. Furthermore, the 1.5-kb LAT RNA detected in SCID trigeminal ganglia after infection with *in*1814 (Fig. 3) is seen only in latently infected tissue (44), strongly suggesting that some cells are latently infected.

It is possible that after the initial infection, as *in*1814 reached the trigeminal ganglia, some of the neurons became latently infected, while in other neurons, the virus replicated and spread to surrounding satellite cells. The difference between these neuronal groups is not clear. The number of particles reaching a cell or a difference in neuronal physiology (23) could both be responsible. Judging from the increasing number of neurons that were LAT positive up to 4 to 6 weeks p.i., it seemed that the generation of latently infected cells was continuous, as a consequence either of viral multiplication and spreading within the nervous system and/or of a continuous supply of virus from the eyes.

Our study indicates that the interaction between HSV-1 and neurons in vivo could be quite similar to those interactions observed in in vitro-cultured neurons. Cultured neurons can be both permissive and restrictive to HSV-1, and the outcome of infection is MOI dependent (53). The observation that some neurons seem to be able to restrict HSV-1 replication in immunodeficient animals suggest that in some neurons, the establishment and maintenance of HSV-1 latency are regulated without an immune component, as was previously proposed by Openshaw et al. (31). However, it should be noted that our results do not exclude the possibility of an alternative active immune regulation of the establishment phase of HSV-1 latency, as suggested by Simmons and Tscharke (41). It is possible that HSV-1 latency can be established in two subsets of neurons: (i) in some neurons (which were perhaps reached by less virus), latency could be established immediately and without any interference from the immune system; (ii) in another subset of neurons (perhaps reached by more virus or physiologically more permissive to HSV-1), some steps in replication might be suppressed by the immune system, resulting in latency instead of cell death (41, 51).

Our finding that HSV-1 *in*1814 can maintain a latent infection in some of the neurons of SCID mice could seem to contradict previous data showing that immunosuppressive treatment of latently infected mice may lead to viral recrudescence, thus suggesting that an intact immune system is a prerequisite for the maintenance of latency (31). These conflicting data, however, can be resolved if we conclude that latency, at least in some neurons, is controlled by nonimmunological (probably neuronal) factors. However, if this cellular control fails and viral reactivation is initiated, the immune system will remove cells in which the replication process has been initiated or will interfere with the initiated replication process.

It should also be noted that although it seems very likely that it is the special physiology of some neurons that makes the establishment and maintenance of HSV-1 latency possible in immunodeficient animals, our present study did not address the role of nonspecific defense factors (i.e., NK cells, macrophages, etc.). These factors are known to be active in SCID mice (reviewed in reference 4) and may have a role in the establishment and maintenance of HSV-1 latency.

In summary, inoculation of SCID mice with HSV-1 *in*1814 resulted in a unique, persistent infection which enabled us to study the molecular biology of virus-host cell interactions involved in the pathogenesis of HSV-1 persistent infections with no or minimal interference by a specific immune response. We found that the restricted pattern of viral gene expression characterizing the latent infection of neurons can occur in the absence of a specific immune response. Our findings presented here help us better understand the pathogenesis of HSV-1 infections and may help us design future strategies against HSV-1 diseases in immunocompromised humans.

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