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Persistence of Viral RNA in Mouse Brains after Recovery from Acute Alphavirus Encephalitis

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Little is known about the relationship between recovery from acute viral encephalitis and the clearance of viral genetic material from the central nervous system. In a mouse model of Sindbis virus encephalitis, we have previously shown that clearance of infectious virus is mediated by antibody-induced restriction of viral gene expression rather than by cytotoxic destruction of virally infected cells. To explore whether Sindbis virus genomes persist in mouse brain after the clearance of infectious virus, we used reverse transcriptase-polymerase chain reaction amplification methods to detect Sindbis virus RNA in brain samples from immunocompetent BALB/c and antibody-treated immunodeficient *scid*/CB17 mice. RNA sequences from both the nonstructural region (NSP1 gene) and structural regions (E2 gene) of Sindbis virus were detected in the brains of all BALB/c and antibody-treated *scid* mice examined at 1, 2, and 3 months after infection. Additional BALB/c mouse brains were also positive at 8, 12, and 17 months after infection. To determine whether persistent RNA was capable of resuming unrestricted replication in the absence of the continuous presence of antiviral antibodies, viral titers were measured in the brains of *scid* mice at 1, 2, 3, and 6 months after antibody treatment. Viral reactivation was seen in *scid* mice treated with hyperimmune serum or a low dose of monoclonal antibody to the E2 envelope glycoprotein, but not in mice treated with a high dose of monoclonal antibody to E2. Replication of infectious virus isolated from *scid* mouse brain could be restricted by repeat treatment with immune serum, indicating that viral reactivation is not due to antibody-escape mutations. These results demonstrate that Sindbis virus can persist long term in a nonproductive form in mouse brain and suggest that the humoral immune response plays an important role in preventing viral reactivation.

Neurons are a favored site for persistence of both productive and latent viral infections. Two essential ingredients for the establishment of persistent infection, the ability of a virus to assume a nonlytic phenotype and to avoid host immunologic surveillance, are often present in viral infections of neurons. Although the precise molecular interactions between viruses and host cells leading to viral persistence are not well defined in most systems, significant advances have been made in our understanding of both latency and immune avoidance in neuronotropic infections. For example, the lack of cellular transcription factors required for expression of early viral gene products may enable herpes simplex virus type 1 (reviewed in reference 10), the prototype of the α or neurotropic class of herpesviruses, to establish latency in neurons. The lack of surface major histocompatibility complex class I expression on neurons may enable viruses such as lymphocytic choriomeningitis virus (16) to evade immunologic surveillance by virus-specific cytotoxic T lymphocytes and establish persistent productive neuronal infection. Other mechanisms that are not restricted to neuroviral infections, such as immunologic tolerance associated with neonatal exposure (Borna disease virus [12], arenaviruses [2, 18], antibody-induced antigenic modulation (measles [8, 9]), or selection of viral genetic mutants, (measles [4, 5, 38]), may also contribute to the ability of certain viruses to avoid the immune system and persist in neurons.

Despite this vulnerability of neurons to persistent viral infections, several arthropod-borne RNA viruses, including the alphaviruses, bunyaviruses, and flaviviruses, cause acute encephalitis that is characterized by the development of an effective immune response and viral clearance in hosts that do not succumb to fatal disease. In immunocompetent animals, latent or persistent productive central nervous system (CNS) viral infection has not been described. The effective clearance of these viruses from neuronal targets that lack surface class I major histocompatibility complex expression suggests that viral clearance may involve a non-cytotoxic-T-lymphocyte-mediated immunologic mechanism. Recently we have shown that Sindbis virus (SV), the prototype alphavirus, can be cleared from the CNS of persistently infected *scid* mice as well as from persistently infected cultured neurons by treatment with monoclonal antibodies (MAbs) to the E2 envelope glycoprotein (19). Antibody acts by directly restricting viral gene expression rather than by mediating cytotoxic destruction of virally infected neurons.

The existence of an antibody-mediated noncytotoxic mechanism for viral clearance raises the possibility that, even in acute CNS viral infections, viral genomes persist in neurons for prolonged periods after the termination of productive infection. In other experimental models, including measles (8, 9, 20) and Aleutian mink parvovirus infection (1), in which antibody against structural viral proteins down-regulates viral gene expression, viral proteins and RNA persist and cause host pathology. In SV infection, no viral RNA is detectable by in situ hybridization within 3 weeks of infection (19), and no neurologic disease is apparent in immunocompetent or antibody-treated *scid* mice after the clearance of infectious virus. However, these findings do not exclude the possibility that RNA persists in quantities that are below the sensitivity of in situ hybridization. The persistence of very low levels of viral RNA after acute viral

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encephalitis could have biologically important consequences, including deleterious effects on neuronal function, viral reactivation in the absence of continuous antibody synthesis, or stimulation of immune-mediated damage (if any viral proteins are translated).

In this study, we explored whether viral RNA persists in mouse brain after recovery from acute encephalitis and whether persistent RNA, if present, can resume unrestricted replication in the absence of continuous antiviral antibody production. To address these questions, we used an experimental model of wild-type SV infection in adult BALB/c and *scid*/CB17 mice. SV is a single-stranded positive-strand RNA virus that replicates predominantly in neurons after intracerebral inoculation in adult mice (14, 15, 29). As previously described (19), immunocompetent mice develop clinically silent, self-limiting encephalitis in which infectious virus is cleared within 8 days after infection. Severe combined immune deficient (*scid*) mice develop persistent infection that can be cleared by the administration of hyperimmune serum (HIS) or MAbs to the E2 envelope glycoprotein. Thus, this model can be used to examine long-term sequelae after recovery from acute, nonfatal viral encephalitis as well as the possibility of viral reactivation in the absence of a sustained humoral immune response.

Using polymerase chain reaction (PCR) amplification methods, we demonstrate that SV RNA persists chronically after recovery from acute SV encephalitis in the brains of immunocompetent BALB/c and antibody-treated *scid* mice. In addition, we show that viral reactivation may occur in antibody-treated *scid* mice, depending on the dose of immunotherapy directed against the critical viral epitope involved in antibody-induced restriction of SV gene expression.

MATERIALS AND METHODS

Virus. SV strain AR339 (American Type Culture Collection), biologically cloned stock SVIA, was grown and assayed by measuring plaque formation in BHK-21 cells. Stock virus contained 10^9 PFU/ml.

Animal experiments. Four- to six-week-old specific-pathogen-free BALB/c mice (Charles River Breeding Laboratory, Inc., Wilmington, Mass.) and *scid*/CB17 mice (Imdyne, San Diego, Calif.) of either sex were used. Mice were anesthetized with methoxyflurane and inoculated intracerebrally (i.c.) with 10^3 PFU of AR339 (or as indicated in Table 2) in 0.03 ml of Hanks balanced salt solution (HBSS). One week after infection, *scid*/CB17 mice were treated intraperitoneally with 0.2 ml of HBSS containing 0.2 mg of protein G-purified immunoglobulin (Ig) from SV HIS or from anti-E2-c MAb 209 ascitic fluid (19, 31), or as indicated in Table 1. At serial time points after infection, mice were anesthetized and exsanguinated. For virus titration and PCR experiments, brains were removed, quick frozen in ethanol and dry ice, and stored at −70°C. For histopathological examinations, mice were perfused with 4% paraformaldehyde before brain dissection.

Virus titrations. Freeze-thawed tissues were used to prepare a 10% homogenate in HBSS. The viral titers of each homogenate were determined by plaque assay titrations on BHK-21 cells.

RNA preparations. Total RNA was isolated from mouse brain tissue by the RNAzol method (TM Cinna Scientific, Inc., Friendswood, Tex.). In brief, approximately 60 mg of brain tissue was homogenized in 600 μl of RNAzol. Chloroform (60 μl) was added, and the samples were placed on ice for 15 min and centrifuged at 4°C for 15 min at 12,000 × *g*.

TABLE 1. Relationship between dose of MAb 209 therapy and incidence of delayed viral reactivation in MAb 209-treated mice with SV infection

MAb 209 dose (mg)[a]	Acute clearance[b]	Viral reactivation[c]
0.2	5/5	0/5
0.1	3/3	
0.05	3/3	5/5
0.01	1/3	
0.001	0/3	

[a] Mice infected with 10^3 PFU of SVIA on day 0 were treated on day 7 with specified amount of MAb 209 in 0.2 ml of HBSS.

[b] Viral titers measured by plaque assay on day 11 after infection (day 4 after mAb 209 therapy). Number of mice without infectious virus recovered from brain/total number of mice examined per group.

[c] Viral titers measured by plaque assay on day 37 after infection (day 30 after MAb 209 therapy). Number of mice with infectious virus recovered from brain/total number of mice examined per group.

The aqueous phase was added to an equal volume of isopropanol, placed at −20°C for 45 min, and centrifuged at 4°C at 12,000 × *g* for 15 min. The RNA pellet was washed twice with 70% ethanol and resuspended in 20 μl of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA). Using this method, approximately 20 μg of RNA per specimen was obtained.

cDNA synthesis. Total cellular RNA (2 μg) was used to synthesize viral cDNA with avian myeloblastosis virus reverse transcriptase (RT) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and random hexamer priming. Random hexamer priming allowed synthesis of cDNA complementary to both plus- and minus-strand viral RNA. Each cDNA reaction contained 2 μl of 10× buffer (0.5 M Tris-HCl [pH 8.3], 0.2 M KCl, 0.1 M $MgCl_2$), 1 μl of 0.1 M dithiothreitol, 40 U of RNAsin (Boehringer Mannheim), 40 U of avian myeloblastosis virus RT, 2 μl of RNA (1 μg/μl concentration), 2 μl of 10 mM deoxynucleoside triphosphate (dNTP) mix, and 2 μl of 10× hexanucleotide mixture (Boehringer Mannheim) in a total volume of 20 μl. Samples were incubated for 45 min at 42°C and then for 5 min at 94°C (to inactivate RT), and 80 μl of distilled H_2O was added. To detect minus- or plus-strand RNA, 100 pM plus-strand or minus-strand SV primer, respectively, was substituted for random hexamers in cDNA synthesis reactions.

PCR amplification of cDNA. cDNA samples were amplified by PCR in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.) by 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Each PCR mixture consisted of 5 μl of GeneAmp 10× PCR buffer (Perkin-Elmer Cetus), 10 μl of 1 mM dNTPs, 2.5 μl of 20 μM primers, 0.25 μl of *Taq* polymerase (Perkin-Elmer Cetus), and 5 μl of cDNA in a total reaction volume of 50 μl. SV nucleotide sequences for oligonucleotide PCR primers were as follows: envelope (E2) glycoprotein, plus strand (8607 to 8626), 5′-GGATCGTCTGGCAGAAGCAA-3′; minus strand (8893 to 8912), 5′-AAGCCTTCTACACG GTCCTG-3′; nonstructural protein 1 (NSP1), plus strand (236 to 255), 5′-CACAGCGACGATCTTGGACA-3′; minus strand, (485 to 504) 5′-ATTCGGCACGCATGTTGCAG-3′. As an internal control for RNA extraction and cDNA synthesis, PCR amplification was also performed on all cDNA samples using primers specific for the constitutively expressed cellular gene, glyceraldehyde phosphate dehydrogenase (GAPDH) (plus-strand, 5′-ACCACCATGGAGAAG GCTGG-3′; minus strand, 5′-CTCAGTGTAGCCCAGG

ATGC-3'). Each PCR product (15 μl) was electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. PCR products were verified by performing Southern blot hybridization with the following ^{32}P-labeled internal oligonucleotide probes: E2 (8730 to 8759), 5'-GTCTGGGA CGAAGCGGACGATAACACCATA-3'; NSP1 (413 to 442), 5'-TAAGGATCTCCGGACCGTACTTGATACGCC-3'; GAPDH, 5'-GTGGAAGGACTCATGACCACAGTCCATGCC-3'.

Histopathology. Brains from paraformaldehyde-perfused mice were embedded in paraffin, cut in 5-μm sections, and stained with hematoxylin and eosin. Immunoperoxidase staining for glial fibrillary acidic protein and phosphorylated neurofilament H was performed with a rabbit polyclonal antibody (Dako Corp., Carpinteria, Calif.) (1:1,000 dilution) and mouse MAb (Sternberger Monoclonals Inc., Baltimore, Md.) (1:2,000 dilution), respectively, using the avidin-biotin-peroxidase method (Vectastain ABC Kit; Vector Laboratories, Burlingame, Calif.) as described previously (14).

ELISA. Serum levels of anti-SV antibody were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (31) with the following modification: ELISA reactivity of each sample was determined by modified probit analysis (11).

DNA sequencing. PCR amplification products of viral cDNA were sequenced by the Genetics Resources CORE Facility of The Johns Hopkins University School of Medicine by the fluorescent dideoxy chain termination method (25). An automated DNA sequencer (model 373A; Applied Biosystems Inc., Foster City, Calif.) was used.

RESULTS

Persistence of SV structural-region RNA. To determine whether any SV RNA persists in mouse brain at times remote from acute infection, we analyzed brains from five BALB/c and five MAb 209-treated *scid* mice at 1-, 2-, and 3-month intervals after intracerebral SV infection. (Clearance of RNA detectable by in situ hybridization occurs between 14 and 21 days after infection in BALB/c mice and 14 and 21 days after antibody treatment in *scid* mice [19]). In initial experiments, we performed RT-PCR to detect SV RNA using primers for the E2 envelope glycoprotein gene which is present in both the full-length genomic 49S RNA and subgenomic 26S RNA species. The results of Southern hybridizations with a ^{32}P-labelled internal oligonucleotide probe are shown in Fig. 1A (data from four of five mice per time point are shown; results are identical for the one mouse per time point not included in Fig. 1A). Negative and positive controls included in Fig. 1 are an uninfected BALB/c mouse and a persistently infected *scid* mouse (day 7 postinfection, no antibody treatment), respectively. As an internal control for RNA extraction and cDNA synthesis, we performed RT-PCR on all brain samples using primers for the constituitively expressed cellular gene GAPDH (Fig. 1C). Figure 1A demonstrates that mouse brain samples from all BALB/c and antibody-treated *scid* mice contained a PCR product of the expected 305-bp size that hybridized with an SV E2 internal oligonucleotide probe. Thus, both immunocompetent and MAb 209-treated *scid* mice had detectable SV E2 RNA in their brains up to 3 months after SV infection.

Persistence of SV nonstructural-region RNA. The data shown in Fig. 1A do not establish whether viral RNA sequences from full-length genomic 49S RNA are present, since E2 sequences are contained in the subgenomic 26S RNA as well as in the full-length genomic 49S RNA.

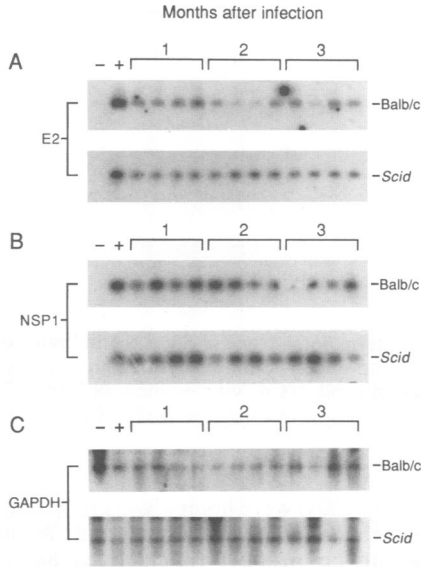

FIG. 1. SV RNA persistence in brains of immunocompetent BALB/c and immunodeficient *scid*/CB17 mice. Southern hybridization analysis of RT-PCR amplification products of viral sequences from E2 gene (305 bp) (A), NSP1 gene (268 bp) (B), or mRNA of constitutive cellular gene GAPDH (508 bp) (C).

Therefore, we performed PCR on the same samples using primers specific for the NSP1 gene, which is present only in the full-length genomic 49S RNA. The results shown in Fig. 1B indicate that viral RNA sequences from full-length genomic 49S RNA are present in all samples from BALB/c and MAb 209-treated *scid* mice at 1, 2, and 3 months after infection (as above, data are shown for four of five mice for each time point).

We attempted to differentiate between plus- and minus-strand RNA by using SV minus- and SV plus-strand complementary oligonucleotide primers for cDNA synthesis rather than the random hexamer primers. (This method of minus-strand detection by PCR has been used for detecting replication activity of poliovirus [24] and hepatitis C virus [30].) However, using SV strand-specific priming for cDNA synthesis and 40 cycles of PCR amplification, we were unable to detect any PCR products for either E2 or NSP1 gene sequences. Substitution of random hexamer with oligo(dT) primers in the cDNA synthesis reaction yielded PCR products which were similar to those shown in Fig. 1A and B, confirming the presence of intact polyadenylated positive-strand SV RNA.

Long-term persistence of E2 and NSP1 genes in BALB/c mice. After determining that immunocompetent BALB/c mice have SV RNA sequences detectable by RT-PCR in their brains 3 months after viral infection, we analyzed brain samples from three additional BALB/c mice that had been infected with SV 8, 13, and 17 months earlier. These three mice (Fig. 2) were also positive for both E2 and NSP1 genes by RT-PCR detection.

Nucleotide sequencing of viral cDNA PCR products. To screen for possible genetic mutations that may play a role in the pathogenesis of viral persistence (and to further confirm the identity of our PCR products), we sequenced E2 and NSP1 PCR products from brain samples of mice 8, 13, and 17 months after infection using the fluorescent dideoxy chain termination method. Sequences for the NSP1 268-bp product

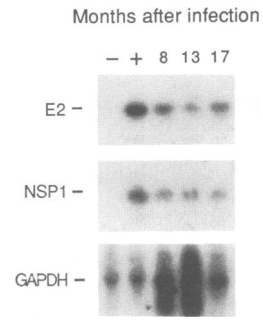

FIG. 2. Long-term persistence of SV RNA in brains of immunocompetent BALB/c mice. Southern hybridization analysis of RT-PCR amplification products of viral sequences from E2 gene (305 bp), NSP1 gene (268 bp), and cellular GAPDH gene (508 bp).

(nucleotides 236 to 504) were homologous to wild-type SVIA in all three specimens. However, nucleotide sequencing of the E2 PCR 305-bp product (nucleotides 8607 to 8912) revealed an A→T substitution at position 8795 in all three specimens, resulting in a glutamine→histidine change at amino acid 55 of E2. This mutation has been observed to occur in neuroadapted SV (22) and has been shown to be an important determinant for age-related neurovirulence (34). The remainder of the E2 region sequenced was homologous to SVIA.

Histopathologic examination of brain sections. To examine whether long-term persistence of SV RNA in immunocompetent BALB/c mice was associated with any histopathologic changes, we obtained brain sections from three BALB/c mice 8 months after SV infection. Routine hematoxylin and eosin staining revealed scattered foci of mononuclear cell infiltration in sections from all three SV-infected mice (Fig. 3), but none in age-matched uninfected control mice. In contrast to the perivascular pattern of infiltration that predominates during acute SV infection, these infiltrates were located intraparenchymally. Despite the presence of inflammatory infiltrates, immunocytochemical staining with a polyclonal antibody to glial fibrillary acidic protein did not reveal any abnormal patterns suggestive of reactive gliosis. To ascertain whether there was any histopathologic evidence of active neuronal injury, we stained brains with an MAb to phosphorylated neurofilament H. In the presence of neuronal injury, immunocytochemical reactivity is observed within neuronal cell bodies, whereas in normal CNS tissue, reactivity is confined to axonal processes (reviewed in reference 37). In the brains of SV-infected mice, no immunoreactivity was seen in neuronal perikarya, and the pattern of staining was similar to that seen in age-matched uninfected mice. Thus, despite the presence of low levels of viral RNA and scattered foci of CNS inflammatory cells, we were unable to detect other histopathologic abnormalities in the brains of BALB/c mice examined 8 months after SV infection.

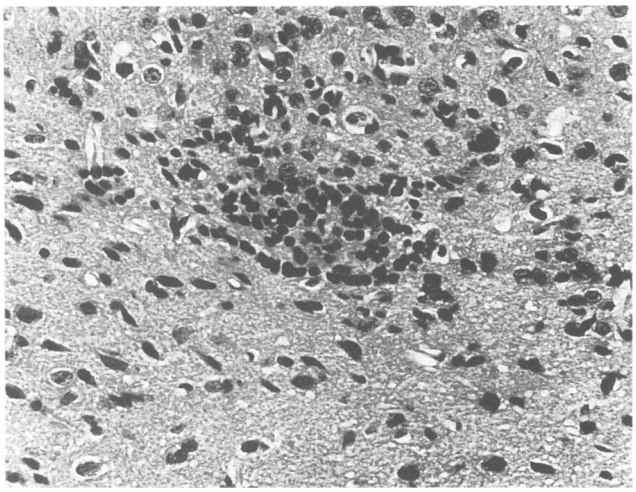

FIG. 3. Hematoxylin-and-eosin-stained sagittal section from BALB/c mouse basal forebrain showing parenchymal lymphocytic infiltration 8 months after SV infection. ×40 magnification.

Recurrence of unrestricted replication after antibody treatment in *scid* mice. Viral titers were measured in brain samples from all BALB/c and MAb 209-treated *scid* mice in the experiment shown in Fig. 1 (five mice per group per time point [1, 2, and 3 months after infection]). An additional five MAb 209-treated *scid* mice were assayed at 6 months after infection. In these experiments, *scid* mice were treated with 0.2 mg of MAb 209 ($\log_{10}$ plaque-reduction neutralization titer = 3.07; $\log_{10}$ ELISA value = 3.9) intraperitoneally 7 days after i.c. inoculation of 10^3 PFU of SVIA. In addition, viral titers were also measured at 30-, 45-, 60-, and 90-day intervals in SV-infected *scid* mice treated with an equivalent Ig protein concentration of SV HIS (three to six mice per time point). Previous studies have shown that treatment of persistently SV-infected *scid* mice with either of these antibody regimens results in clearance of infectious virus from brain within 48 h (19) (and viral titers from five mice in each treatment group obtained 2 days after therapy are included in the results of present experiment). The percentage of mice at each time point from whom infectious virus could be recovered by plaque assay titration is shown in Fig. 4A. The mean ELISA anti-SV antibody titers in serum for each mouse group at each time point are shown in Fig. 4B. Immunocompetent BALB/c mice had no CNS viral reactivation and had sustained high levels of serum anti-SV antibody. Between 1 and 3 months after treatment, CNS viral reactivation occurred in 33 to 66% of *scid* mice treated with SV HIS. In contrast, SV reactivation did not occur in any of the *scid* mice monitored up to 6 months after treatment with MAb 209, and at 6 months after treatment, *scid* mouse sera contained no reactivity with SV.

To determine whether the discrepancy between the incidence of viral reactivation in those mice treated with MAb 209 versus those mice treated with SV HIS could be explained by the different concentrations of Ig molecules to the E2-c epitope that are specifically required for inhibition of intracellular SV replication, we performed additional clearance experiments with various doses of MAb 209. These are summarized in Table 1. First, we determined the lowest possible dose of MAb 209 which would reliably result in the acute clearance of infectious virus from *scid* mice persistently infected with SV. Then, we administered this dose (0.05 mg of MAb 209) to five additional SV-infected *scid* mice and measured viral titers 30 days later. All five mice were positive for infectious virus on day 30, confirming that the dose of MAb to E2-c is a critical determinant of whether unrestricted viral replication (defined by recovery of infectious virus) is reversibly or irreversibly inhibited by anti-SV antibodies.

To ascertain whether viral reactivation occurred because of mutations in SV that resulted in resistance to immune

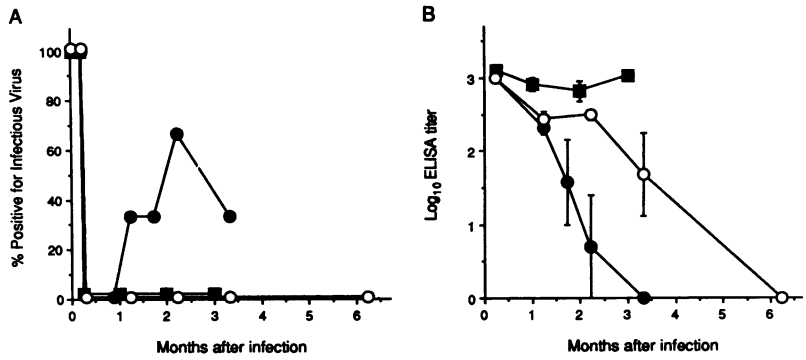

FIG. 4. CNS SV reactivation after antibody treatment in *scid*/CB17 mice. Mice were infected with 10^3 PFU of SV i.c. on day 0 and treated intraperitoneally with 0.2 mg of MAb 209 (○) or 0.2 mg of SV HIS (●) on day 7. Normal BALB/c mice controls (■) were infected with 10^3 PFU of SV i.c. on day 0. (A) Percentage of mice with infectious virus in brain measured by plaque assay titration at serial time points after infection. Five mice per group are included for BALB/c mice and MAb 209-treated *scid*/CB17 mice. Three to six mice per group are included for SV HIS-treated *scid*/CB17 mice. (B) Mean serum anti-SV antibody titers measured by ELISA in the same groups of mice as in panel A.

serum-induced restriction of viral replication, we infected additional *scid* mice with brain homogenates containing infectious SV from *scid* mice previously treated with SV HIS and determined whether repeat treatment with SV HIS would result in clearance of infectious virus. These results are summarized in Table 2. Inoculation of five brain homogenates into new *scid* mice resulted in persistent productive SV infection as indicated by recoverable infectious virus in control mice who were not treated with HIS. Treatment of these *scid* mice with SV HIS resulted in clearance of infectious virus, indicating that these virus strains were not antibody-escape mutants. (An additional brain homogenate, 256B, resulted in 100% mortality in the *scid* mice before day 7, and therefore the effect of treatment of HIS was not determined.) In addition, the pattern of ELISA reactivities of all five viruses in Table 2 and 256B with a panel of six MAbs to E2 does not differ from that of wild-type SVIA (data not shown). Taken together, these results suggest that the viral reactivation is not due to mutations in E2 that permit the virus to escape antibody-induced restriction of viral replication.

DISCUSSION

SV, a positive-strand RNA virus that causes acute nonfatal encephalitis in adult mice, provides an excellent animal model for studying interactions between the humoral immune response and CNS viral replication. Previously, we have shown that in the absence of humoral immunity, SV causes persistent productive infection in rodent neurons that can be terminated by treatment with MAbs to the E2 envelope glycoprotein (19). In the present study, we demonstrate that in the presence of an effective humoral immune response that clears infectious virus from mouse brain in acute infection, SV genomes persist long term in a nonproductive form. In antibody-treated *scid* mice, viral reactivation may occur, implying that sustained humoral immunity may be required to maintain nonproductive infection.

TABLE 2. Effect of hyperimmune serum therapy on clearance of infectious SV isolates from *scid* mice with CNS SV reactivation

Viral isolate[a]	Treatment[b]	No. of mice	Mean viral titer[c] ($\log_{10}$ PFU/g of brain)
348	SV HIS	3	<1.7
	HBSS	1	4.93
359	SV HIS	3	<1.7
	HBSS	1	6.04
379	SV HIS	3	<1.7
	HBSS	1	3.80
381	SV HIS	3	<1.7
	HBSS	1	4.67
393	SV HIS	3	<1.7
	HBSS	1	3.83

[a] *scid* mice treated with 10^3 PFU of whole-brain homogenate i.c. on day 0.
[b] *scid* mice treated with 0.2 mg of SV HIS (in 0.2 ml of HBSS) or 0.2 ml of HBSS on day 7.
[c] *scid* mice were sacrificed on day 11, and viral titers were measured by plaque assay titration.

The mechanism by which positive-strand viral RNA genomes can persist intracellularly in vivo without active replication is not known. Abnormal regulation of minus-strand production has been postulated to play a role in persistence of nonproductive enteroviral infections in muscle (6, 17) and Theiler's virus in oligodendrocytes (3). Cunningham et al. (6) have proposed that the equimolar synthesis of plus and minus strand observed in persistent nonproductive enteroviral infection (compared with the large excess of plus strand seen in productive infection) may inhibit the translation of virus-specific gene products, thus preventing the production of progeny virus particles. In addition to blocking the translation initiation site, minus strand could act by inhibiting transcriptional activity or by enhancing degradation of message-sense viral RNA (the latter mechanism has been shown to be important in antisense oligonucleotide inhibition of gene expression [reviewed in reference 32]). Interestingly, the synthesis of antisense RNA latency associated transcripts overlapping with the 3′ end of the immediate-early gene ICP0 transcription unit has been invoked as the molecular basis for latency of herpes simplex virus type 1 infection (reviewed in references 13 and 33). Unfortunately, the levels of SV RNA in the mouse brains in our study were too low to permit PCR amplification by strand-specific priming in our RT reaction, and thus, we were not able to assess the role of minus-strand production in the establishment of nonproductive infection.

Other mechanisms postulated to be involved in the establishment of nonproductive RNA viral replication include the generation of defective interfering particles, biased somatic hypermutation, and the induction of cellular interferon synthesis. Although alphavirus defective interfering particles isolated from in vitro tissue culture systems have been

studied extensively (reviewed in reference 26), a biologically important role for defective interfering particles in vivo has not been established. Given the uniform reversibility of nonproductive replication in *scid* mice treated with a low dose of MAb 209, it seems unlikely that defective interfering particles are of primary importance in the maintenance of latent CNS SV infection in vivo. In persistent measles virus infection of the CNS (subacute sclerosing panencephalitis), hypermutation of the M protein (and possibly other viral proteins) may affect virus assembly and result in nonproductive infection (4, 5, 38). Nucleotide sequencing of a larger segment of the SV genome will be required to address the possibility that biased hypermutation is important in SV persistence; the homology observed between the regions of NSP1 and E2 genes sequenced in SV-infected mice in our study and that of wild-type SV is preliminary evidence arguing against such a mechanism. The single nucleotide change (an A→U change at position 8795 in the E2 envelope glycoprotein gene) in viral RNA isolated from persistent nonproductively SV-infected BALB/c mice is unlikely to play a role in the pathogenesis of nonproductive replication, as an identical nucleotide change is also found in viruses isolated from persistent productively SV-infected *scid* mice (17a). Furthermore, the absence of detectable viral proteins in persistent nonproductive SV infection contrasts markedly with subacute sclerosing panencephalitis and suggests that the defect in production of progeny virus is not a primary consequence of defective viral assembly, but rather of transcriptional or translational inhibition. In addition, it is also unlikely that the high levels of interferon measured in SV encephalitis (29) are important in inducing nonproductive replication; preliminary data in cultured rat neurons suggests that antibody-induced restriction of SV gene expression occurs in the absence of any detectable interferon production (17a). Thus, the mechanisms involved in the establishment of nonproductive SV replication remain undefined. Elucidation of the molecular events in antibody-induced restriction of SV gene expression is likely to provide important clues.

Given the absence of detectable viral proteins and extremely low levels of viral RNA (detectable only by PCR and not by in situ hybridization), the question arises as to whether any degree of viral replication occurs in persistent nonproductive CNS SV infection. It seems implausible that an RNA virus lacking reverse transcriptase could persist intracellularly for months without some degree of replicative activity. The concept of restricted viral replication is supported by two observations that suggest that limited translation of viral proteins also occurs. In this study, immunocompetent BALB/c mice had persistent CNS inflammation 8 months after the clearance of infectious virus. Similarly, Tyor et al. (35) have demonstrated that SV-specific Ig-secreting B cells can be recovered from mouse brain 12 months after acute infection. Thus, continued viral antigenic stimulation is likely to be present, although long-term CNS retention of SV-specific B lymphocytes without continued antigenic stimulation cannot be ruled out.

The data from this study do not permit us to definitively conclude whether SV persists in an abortive (defined as irreversible restricted replication) as well as latent (defined as reversible restricted replication) form. In *scid* mice treated with HIS or low-dose MAb to E2, viral reactivation occurs in some mice, demonstrating that SV can persist in a latent form. In *scid* mice treated with a higher dose of MAb to E2, no viral reactivation was observed at 6 months after infection, suggesting that higher doses of MAb induce an abortive infection. However, despite the absence of detectable serum antibody in these mice, we cannot exclude the possibility that passively transferred antibody was still present in the CNS and that if observed long enough, viral reactivation would eventually occur in these mouse brains as well. In immunocompetent BALB/c mice, viral reactivation does not occur. The continuous intraparenchymal production of SV-specific IgG that can restrict replication, rather than the induction of an abortive cycle, could explain this phenomenon. However, previous experiments to recover infectious virus from BALB/c mouse brain several weeks after infection by cocultivation, inoculation into suckling mouse brain, or treatment with immunosuppressive agents have been unsuccessful (29a). Taken together, these observations suggest, but do not prove, that transient or low-dose immunotherapy induces latency, whereas natural humoral immunity or high-dose immunotherapy induces abortive infection.

The biologic consequences of persistent low levels of viral RNA in brain remain to be elucidated. In our study, we could not detect any clinically apparent neurologic abnormalities nor histopathologic evidence of neurologic disease. Further studies are required to address the effects of persistent viral genomes on neuronal function at a molecular level. Neurotransmitter or neurohormonal synthesis or other specialized cellular functions could be perturbed without direct cell damage, leading to subtle abnormalities in CNS function. In persistent productive neuronal infection with lymphocytic choriomeningitis virus, mouse brain growth hormone (36) and somatostatin mRNA (21) levels may be selectively reduced in the absence of morphologic evidence of CNS injury. Similar effects on higher cellular functions could theoretically also occur with persistent nonproductive viral infection. In addition to direct effects of persistent viral genomes on neuronal function, any translation of viral proteins could also provide important antigenic stimuli for immune-mediated CNS inflammatory diseases.

Our findings support the hypothesis that reactivation of latent RNA viral genomes in the presence of waning humoral immunity may be involved in the pathogenesis of neurologic syndromes that follow recovery from acute viral encephalitis. Examples include the postpolio syndrome (27), recurrent neurologic disease after Japanese encephalitis (28), and the late neurologic syndrome associated with immune plasma treatment for Argentine hemorrhagic fever (Junin) virus (23). Sharief et al. (27) have recently postulated that postpolio syndrome, which occurs decades after initial infection and may involve new muscle groups, results from reactivation of persistent viral infection, as evidenced by intrathecal poliovirus-specific IgM synthesis and increased soluble interleukin-2 receptor in the cerebrospinal fluid. A prospective study of children monitored after an initial episode of Japanese encephalitis revealed the presence of Japanese encephalitis virus-specific IgM antibodies and a new rise in Japanese encephalitis virus hemagglutination-inhibition antibodies exclusively in children who developed recurrent neurologic symptoms (28). In the late neurologic syndrome associated with Argentine hemorrhagic fever virus infection, recurrence of neurologic symptoms occurs 4 to 6 weeks after treatment with immune plasma (23) and is associated with delayed seroconversion and intrathecal synthesis of anti-Junin virus antibodies (7). One possible explanation is that CNS viral reactivation occurs after the clearance of exogenously administered antiviral antibodies in patients whose endogenous humoral immune responses have been suppressed. Additional studies, including the direct demonstra-

tion of viral genomes in the CNS, will be required to fully address the role of RNA viral persistence in the pathogenesis of these neurologic syndromes.

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