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The role of host immunocompetence in neuroinvasion of Sindbis virus

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Summary. Viral infections of the central nervous system (CNS) following peripheral inoculation of Sindbis viruses were studied. The use of viral strains, which vary in their neuroinvasive and neurovirulent properties, and various strains of mice, which differ in immunocompetence, revealed several pathways of viral neuroinvasion in adult mice. A genetic-trait dependent mechanism was exhibited by the neuroinvasive viruses, showing a similar pattern in all mice strains tested. A second mechanism, dependent on a prolonged high viral load, was exhibited by a noninvasive variant in Severe Combined ImmunoDeficient (SCID) mice. The absence of antiviral antibodies in SCID mice allowed the maintenance of a long-term high viremia, leading to a random entry to the CNS and proliferation in brain tissue. An additional pathway for neuroinvasion was induced upon disruption of the blood-brain barrier activity by exogenous reagents and was demonstrated in cases of short lived high viremia of noninvasive viruses.

Introduction

Neuroinvasiveness is defined as the ability of a neurotropic virus to invade the host CNS following its introduction by a peripheral route [8, 16, 31]. The two major pathways mediating this spread are via the bloodstream and via nerves. Neuroinvasiveness is considered an inherent trait. Examples of minor changes in the viral genetic material that differentiate a neuroinvasive from a noninvasive virus strain support this notion [12, 28, 30, 32]. Invasion into the CNS by most members of Alphaviridae is considered to be carried out via the hematogenous pathway and is usually linked to the duration of viremia and its levels [23, 24, 29]. However, examples of high viremia without neuroinvasion [1] and vice versa [22], seem to contradict this general correlation. To elucidate the mechanisms

of neuroinvasiveness and to evaluate the contribution of viremia to them, we generated several strains of Sindbis virus which differed in their neuroinvasive and neurovirulent properties [19].

Sindbis virus (SV), among the least pathogenic of the alphaviruses, replicates in muscle cells causing myositis, followed by viremia which may spread the virus in suckling mice to the CNS [15]. The genetic determinants responsible for the neurovirulent traits of the Sindbis virus have been described previously [21, 25].

A strain of SV, isolated from a pool of culicine mosquitoes gathered in southern Israel in 1983, was used as a source for variants which differ in their neuroinvasive and neurovirulent properties in adult mice (summarized in Table 1 and demonstrated throughout this study). Two non-virulent strains were plaque-purified: SVA – a strain that did not invade the CNS when injected peripherally, and SVB – a neuroinvasive counterpart, which penetrated the brain when inoculated by the same route. Both strains proliferated similarly in the brain when injected intracerebrally (i.c.) without causing overt disease symptoms, and were cleared from the system by day 6 after inoculation. The strains SVN and SVNI were isolated by serial passages of the original non-neurovirulent SV in suckling and weanling mouse brain, and finally plaque-purified. Both strains were neurovirulent in weanling mice when injected i.c., however only SVNI was neuroinvasive, as determined by peripheral inoculation.

The variants kept similar patterns of proliferation in suckling mice (2 days old) when inoculated either i.c. or intraperitoneally (i.p.), causing the death of the newborn mice at the same time. Furthermore, in vitro studies showed that the growth in mosquito and vertebrate cell cultures were similar [19].

The genetic differences underlying the variations in the neuroinvasive properties of the SV strains were studied and mapped. In the non-neurovirulent SVA/SVB pair, a single difference in the E2 glycoprotein gene was found to be responsible for the neuroinvasive properties of SVB, whereas, for the neurovirulent SVN/SVNI pair, determinants were identified in both the 5′ non-coding region (5′NCR) and the E2 gene. These data are in accordance with the claim that neuroinvasiveness is a genetic viral trait [5]. To further investigate the differences between the neuroinvasive and non-neuroinvasive strains of SV, we have studied the outcome of infections with the four viral strains in immunocompetent, athymic nu/nu and SCID mice.

Materials and methods

Mice

Female CD-1 mice were obtained from Charles River (London, U.K.). Female BALB/c, CB-17, BALB/c-nude and SCID/CB-17 mice were obtained from IFFA Credo (France). Four week old mice were used throughout the study.

Viruses and cell cultures

Variants of Sindbis virus used in this study were previously described [19]. The two neurovirulent variants, SVN (non-neuroinvasive) and SVNI (neuroinvasive) have been isolated by serial passages of a SV strain in suckling and weanling mice brains. The two non-neurovirulent

variants, SVA (non-neuroinvasive) and SVB (neuroinvasive), have been isolated by plaque purification from the same original SV strain. The Vero cell line, derived from kidneys of normal African Green monkeys, was grown in Dulbecco's Modified Eagle Medium containing 2% fetal bovine serum.

Viremia and virus titers in mice brains

At intervals after inoculation, blood samples were withdrawn from the tail vein of the infected mice, and individual brains (at least, 3 per time point) were processed to produce 20% homogenates in PBS.

Titration of virus in tissue cultures

The virus levels were determined by the original plaque assay on Vero cells [6]. A dilution of virus was added to Vero cell monolayers in tissue culture dishes and incubated at 37 °C for 1 h to permit viral adsorption. The monolayer was overlaid with MEM × 2 and tragacanth (Gum tragacanth Grade III G-1128, Sigma) containing 2% fetal bovine serum and 2.4% $NaHCO_3$. The cultures were incubated (37 °C, 5% CO_2) for 48 h. Plaques were counted after staining the monolayer with neutral red (0.05%).

In vivo assay

Virus lethality was determined by intraperitoneal (i.p.) injection of the virus (0.2 ml) or by intracerebral (i.c.) injection (0.03 ml). The titer of the virus was calculated according to the cummulative mortality observed during 14 days [27].

Reconstitution of SCID/CB-17 mice

To reconstitute the immune system of SCID mice, 2×10^7 spleen cells from uninfected CB-17 mice were injected i.v. into SCID/CB-17 mice, 6 days prior to virus inoculation.

Antibody titers

An enzyme linked immunosorbent assay was used to determine anti-Sindbis IgG and IgM levels. Results are the mean value obtained from sera of 6 mice and represent the highest reciprocal dilution showing a significant positive signal as compared to control serum.

Results

Neuroinvasion of SVA and SVB in immunocompetent mice

Groups of mice (CD-1, BALB/c, CB-17, BALB/c nude and SCID/ CB-17) were inoculated i.p. with 10^4 PFU of SVA and SVB strains of Sindbis virus. SVA and SVB showed similar patterns of viremia in CD-1 mice, peaking at 24 h p.i. and disappearing on day 3 (Fig. 1a). In the brain, however, while SVA could not be detected, the presence of SVB was clearly demonstrated from day 3 until day 7 (Fig. 1b). Two inbred mouse strains, BALB/c and CB-17 (parental strains of the SCID mice), were compared with the outbred CD-1 strain, and no detectable differences in the time course of the infection were found. Similar patterns were found in nude mice (Fig. 1a and 1b), in which viremia of both viruses disappeared on day 4, SVB virus was present in the brain from day 4 till day 9, and SVA could not be detected in the CNS. These results indicate that T cells were not directly

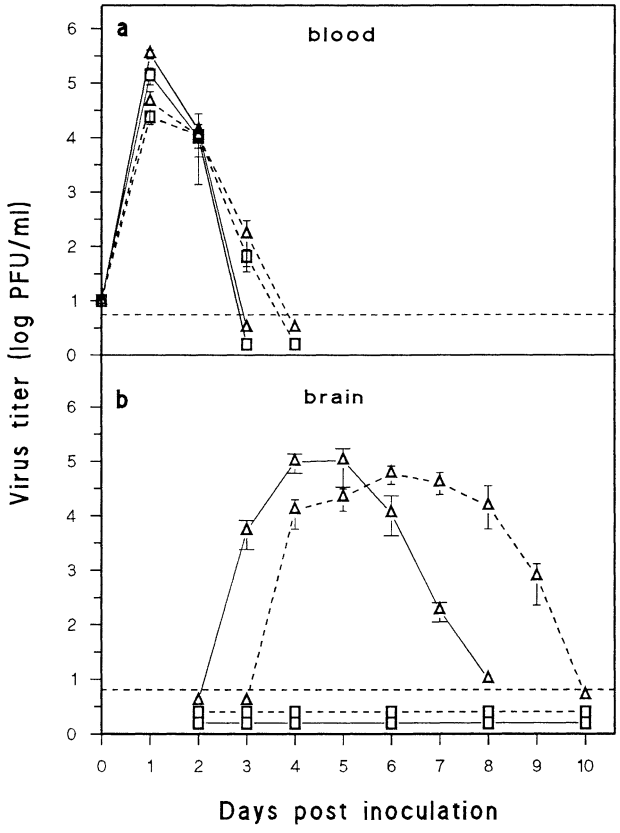

Fig. 1. Sindbis viruses (SVA and SVB) in blood and in brains of CD-1 and BALB/c nude mice. Groups of 4 weeks old female weanling CD-1 and nude mice were inoculated i.p. with 10^4 PFU of SVA or SVB strains of Sindbis virus. Mice were killed at serial time points after inoculation. The amount of virus present in blood and in 20% tissue homogenates of brain was determined by plaque assay titration on Vero cells. Each point represents the arithmetic mean (± SEM) of viral titers (in plaque-forming units per ml of tissue) from three mice. Horizontal dashed line represents the lower limit of virus detection. **a** Growth curves of SVA (□) and SVB (△) in blood of CD-1 mice (solid line) and of nude mice (dashed line). **b** Growth curves of SVA (□) and SVB (△) in brains of CD-1 mice (solid line) and of nude mice (dashed line)

involved in the main mechanisms mediating either neuroinvasion or clearance of the viruses.

Neuroinvasion of SVA and SVB in immunocompromised mice

In SCID mice, which are deficient in T and B cells, different patterns of behaviour were found for both viruses in blood and brain (Fig. 2). The viremia of SVA and SVB rose to maximal levels 24 h after inoculation, and stayed at that level thereafter (tested up to 60 days). SVB was detected in the brain on day 3 (similar to what had been found in CD-1 and CB-17 mice), and no decline was seen thereafter. SVA virus was not found in the brain prior to day 7 p.i., then rising to a similar level as SVB and showing no decrease in titer (tested up to 60 days). The

Table 1. Pathogenicity of Sindbis virus strains in adult susceptible mice

Virus	Mode of inoculation			
	i.c.		i.p.	
	proliferation in the brain	induction of death	invasion into the brain	induction of death
SVA	+	−	−	−
SVB	+	−	+	−
SVN	+	+	−	−
SVNI	+	+	+	+

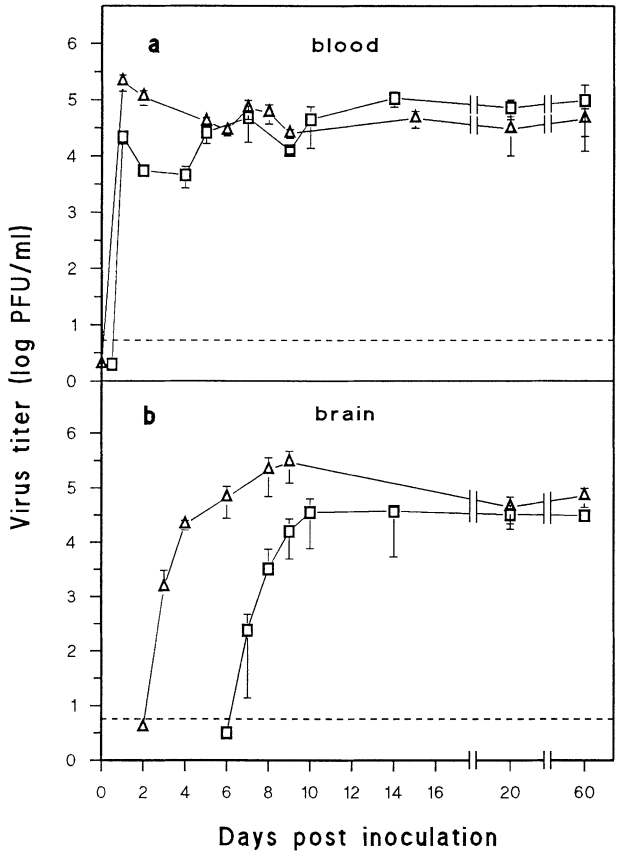

Fig. 2. Sindbis viruses (SVA and SVB) in blood and in brains of SCID mice. Groups of 4 weeks old female SCID/CB17 mice were inoculated i.p. with 10^4 PFU of SVA or SVB strains of Sindbis virus. Mice were killed at serial time points after inoculation. The amount of virus present in blood and in 20% tissue homogenates of brain was determined by plaque assay titration on Vero cells. Each point represents the arithmetic mean (± SEM) of viral titers (in PFU/ml tissue) from three mice. **a** Growth curves of SVA (□) and SVB (△) in blood of SCID mice. **b** Growth curves of SVA (□) and SVB (△) in brains of SCID mice

viruses isolated from the SCID brains were injected into CD-1 mice, and were shown to retain their original characteristics.

The results support previous observations on alphaviruses in SCID mice, indicating that clearance of the viruses from the CNS is mediated by antibodies [7, 9, 18]. This conclusion is further supported by findings presented in Table 2, which show that anti-virus IgM could be detected 3–4 days after inoculation (for CB-17, CD-1 and nude mice), and anti-Sindbis IgG detected a few days later (although in the nude mice, the titer found was 10 folds lower – causing a one day delay in the clearance of the virus from the brain). When the antibodies were not produced, as in the SCID mice, high viremia persisted indefinitely. Reconstitution of the immune system of the SCID mice by administration of naive B and T cells (2×10^7 spleen cells of CB-17 mice injected i.v. 6 days prior to virus injection), enabled the elicitation of anti-viral antibodies, clearing the virus from the system. Therefore, the pattern of distribution of SVA and SVB in blood and brain of the reconstituted SCID mice was similar to that found in CB-17 (Table 2).

The prolonged state of elevated viremia in the SCID mice can be considered as the cause of SVA penetration into the CNS from day 7 p.i. and thereafter. This invasion seemed to be a "random" event (hence the variation in penetration timing), in contrast to the SVB neuroinvasion, where invasion occurred a short-time after the viremia peaked, both in immunocompetent and deficient mice. These data are in accordance with the claim that neuroinvasion in SVB is a genetic viral characteristic, which can be abolished by changing of a single amino acid (position 55) in the E2 glycoprotein [5].

Neuroinvasion of SVN and SVNI in immunocompetent and immunodeficient mice

To further demonstrate the role of the prolonged high viral load in neuroinvasion, we studied an additional pair of Sindbis viruses, the neurovirulent neuroinvasive strain – SVNI, and its noninvasive counterpart – SVN. Both viruses will cause encephalitis and death in CD-1, CB-17 and SCID when injected i.c., even at very low doses. Following i.p. inoculation, SVN and SVNI caused similar viremia in both CD-1, CB-17 and SCID mice, peaking on day 1 p.i. with clearance by day 3 (Fig. 3a). Notably, in SCID mice SVN viremia persisted in the blood at a very low titer for at least 22 days (isolated SVN from the blood at this stage was characterized and no change in neuroinvasive and neurovirulent traits could be detected). SVNI was found in the CNS of either CD-1 or SCID mice by day 3, peaking on days 5–6, eventually causing the death of the mice. SVN could not be detected in the CNS of either CD-1 or SCID mice during the 22 days of the experiments (Fig. 3b). The data presented here showed that although no difference was detected in the viremia caused by either of the two viruses in the two strains of mice, only one of them, the neuroinvasive SVNI, invaded the brain 3 days p.i., suggesting an 'active' mechanism induced by this virus, confirming its genetic properties. This characteristic of SVNI, in comparison with SVN, was attributed to determinants in the 5′ non coding region and/or the E2 gene [5].

Table 2. Comparison of SVA and SVB in blood and in brain of CB-17, SCID and reconstituted SCID mice

Mouse strain		CB-17		SCID		Reconst. SCID	
Virus		SVA	SVB	SVA	SVB	SVA	SVB
Blood[a]	Day 1 p.i.	$1.6\pm0.6 \times 10^5$	$3.4\pm1.4 \times 10^5$	$2.2\pm0.8 \times 10^4$	$2.1\pm0.7 \times 10^5$	$9.4\pm5.4 \times 10^4$	$2.0\pm1.0 \times 10^5$
Blood[a]	Day 3 p.i.	<10	<10	$5.6\pm3.2 \times 10^3$	$7.9\pm2.9 \times 10^4$	60±45	63±38
Brain[b]	Day 4 p.i.	<10	$1.8\pm1.2 \times 10^5$	<10	$2.1\pm0.5 \times 10^4$	<10	$5.2\pm3.0 \times 10^4$
Brain[b]	Day 8 p.i.	<10	<10	$3.2\pm1.6 \times 10^3$	$2.1\pm1.0 \times 10^5$	<10	57±46
IgM[c]	Day 4 p.i.	640	640	<10	<10	320	320
IgG[c]	Day 14 p.i.	25000	25000	<10	<10	20000	20000

[a]Virus level - PFU/ml
[b]Virus level - PFU/ml of 20% brain homogenate
[c]Titer of antiviral antibodies determined by ELISA

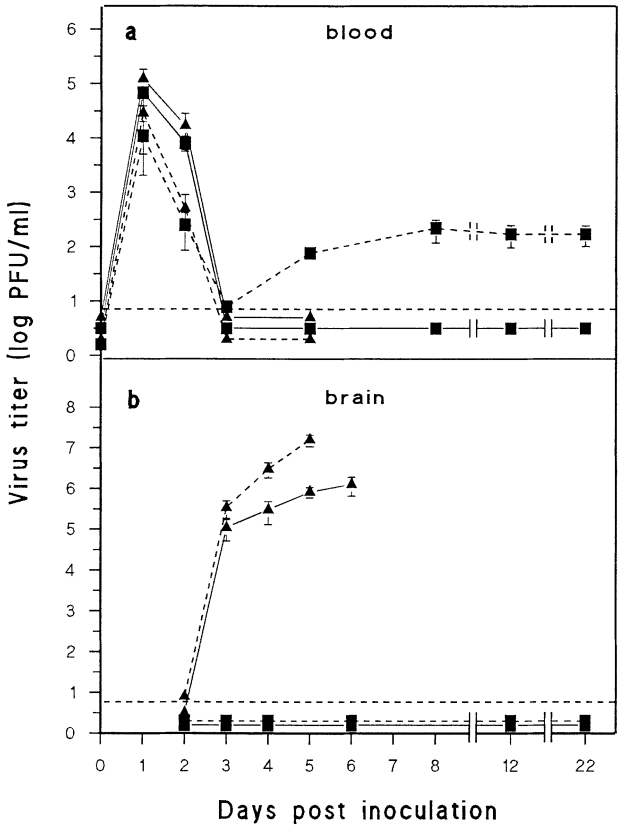

Fig. 3. Sindbis viruses (SVN and SVNI) in blood and in brains of CD-1 and SCID mice. Groups of CD-1 and SCID mice, 4 weeks old, were inoculated i.p. with 10^4 PFU of SVN and SVNI strains of Sindbis virus. Mice were killed at serial time points after inoculation. The amount of virus present in blood and in 20% tissue homogenates of brain was determined by plaque assay titration on Vero cells. Each point represents the arithmetic mean (± SEM) of viral titers (in PFU/ml tissue) from three mice. **a** Growth curves of SVN (■) and SVNI (▲) in blood of CD-1 mice (solid line) and of SCID mice (dashed line). **b** Growth curves of SVN (■) and SVNI (▲) in brains of CD-1 mice (solid line) and of SCID mice (dashed line)

On the other hand, although SVN infection caused a short-lived high viremia in the SCID mice (in contrast to the persistent high viremia caused by SVA), it could not penetrate efficiently into the CNS. We hypothesized that the persistent low viremia shown by SVN in SCID mice did not produce a sufficient viral load to allow neuroinvasion.

Viral neuroinvasion induced by BBB modulation

We tested the possibility that in case of a short-lived high viremia, artificial modulation of BBB permeability would enable viral neuroinvasion. 24 h after SVN i.p. inoculation of CD-1 mice, when viremia was about 10^5 PFU/ml, treatment with reagents known to disrupt BBB integrity, like glycerol, CO_2 or DMSO [11, 17, 26], led to SVN neuroinvasion, encephalitis and death (Table 3). Furthermore,

Table 3. Effect of modulators of blood-brain barrier activity on SVN neuroinvasion

Modulator[e]	Mortality dead/total	%	PFU/brain (on day 5 p.i.)
Control	0/8	0	<10
DMSO[a]	6/8	75	$6.5\pm0.8\times10^5$
CO_2[b]	7/8	87.5	$9.1\pm1.1\times10^5$
Glycerol[c]	6/8	75	$7.0\pm0.9\times10^5$
LPS[d]	5/8	62.5	$8.7\pm0.9\times10^5$

Modulators were administered 24 h after SVN i.p. inoculation in CD-1 mice, when the viremia was $\approx10^5$ PFU/ml. Modulating treatments were as follows:

[a]0.2 ml of 13% DMSO solution in PBS were injected into the tail vein

[b]Mice were exposed to a 30% CO_2 atmosphere for 3 min

[c]0.2 ml of 30% glycerol solution in PBS were injected into the tail vein

[d]LPS (005/B5 Difco Laboratories Inc., Detroit MI) was injected intraperitoneally (100 μg/mouse)

[e]Non-infected mice receiving the modulating treatments did not show any overt effect

administration of lipopolysaccharide (LPS), previously shown to induce the production of soluble factors which enable the penetration of noninvasive viruses [20], also facilitated CNS invasion by SVN (Table 3).

Similarly, BBB modulators can induce viral neuroinvasion when a passive viremia is produced by i.v. inoculation of 10^5 PFU/mouse (LPS-induced neuroinvasion of SVA is shown in Fig. 4).

Discussion

Our unique Sindbis virus strains (Table 1), which were genetically analysed [5], enabled us to study the different pathways of viral neuroinvasion in adult mice. The different pathways of neuroinvasion described in this study are demonstrated by use of SVA and SVB (which differs from SVA by a single amino acid in the E2) and shown in Fig. 4. The time-course of SVA appearance and proliferation in the brain following passive viremia and LPS administration (BBB modulation) are very similar to the pattern obtained after i.c. inoculation, demonstrating an immediate effect of LPS, breaching the BBB. A delayed appearance of the virus in the brain was found following intranasal instillation of SVA. This delay may represent the time required for the retrograde axonal transport to take place.

The natural pathway of neuroinvasion is manifested by SVB (and SVNI) following peripheral inoculation, and is dependent on the genetic neuroinvasive- trait

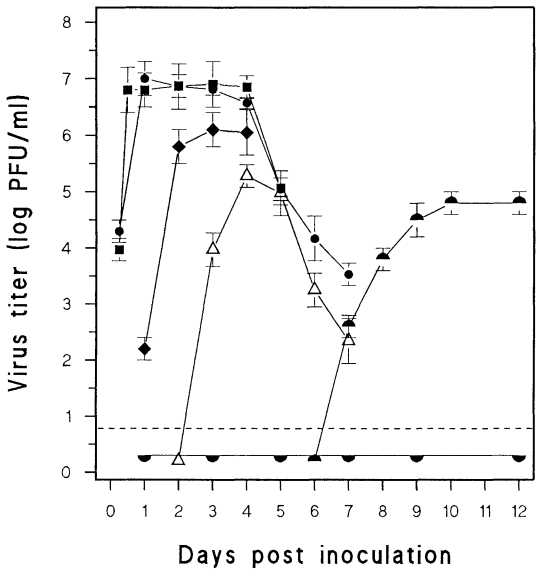

Fig. 4. Different pathways of neuroinvasion of SVA and SVB-viruses. Groups of mice, 4 weeks old were inoculated with 10^4 PFU of each of the viruses.Three groups of CD-1 mice were inoculated either i.p., ic., or intranasal (in) with SVA. A forth group was injected ip with 50μg of LPS immediately prior to SVA inoculation into the tail vein (iv). A fifth group of CD-1 mice was inoculated ip with SVB. SVA was inoculated ip into a group of SCID mice. Mice were killed at serial time points after inoculation and the virus titer present in 20% brain homogenate was determined by plaque assay titration on Vero cells. Each point represents the arithmetic mean (± SEM) of viral titers (in PFU/ml tissue) from three mice. In CD-1 mice : SVA (ic) ●; SVA (in) ◆; SVA (iv or ip) ◒; LPS (ip) followed by SVA (iv) ■; SVB (ip) △. In SCID mice: SVA (ip) ◓

of the virus and the establishment of viremia. This pathway is not influenced by the immune response of the inoculated mice towards the infection and consequently, one given pattern is found in the immunocompetent and the immunocompromised mice.

The last pathway for neuroinvasion, demonstrated in this report (and shown in Fig. 4) seems to be based on a "random infiltration" into the CNS. This mechanism seems to be a random entrance (taking place much later), depending on a prolonged elevated viremia, which will maintain an excessive number of virions in the blood, 'awaiting' entrance. This type of viremia-dependent entrance could be mediated by axonal transport through the olfactory or the trigeminal nerves, as shown for Venezuelan equine encephalitis virus [4].

The specific set of conditions, namely, high and prolonged viremia, required for the 'random neuroinvasion' phenomenon to take place, were obtained for SVA only in the severely immunocompromised host, the SCID mice (Fig. 2). The residual low level viremia shown by SVN in the SCID mice from day 5 onwards (Fig. 3a), was not sufficient to promote efficient passive infiltration, showing that enabling neuroinvasion is not an inherent characteristic of the SCID mice.

In cases of short-lived high viremia of a non-invasive virus an additional stimulus causing modulation of the blood brain barrier is required for viral invasion of the CNS. Such stimuli can be achieved by administration of LPS, glycerol, DMSO and CO_2, reagents previously shown to facilitate CNS penetration of normally noninvasive viruses (Table 3).

A pattern of behavior similar to SVB was demonstrated for the nonvirulent A7(74) strain of Semliki Forest virus. This virus causes high viremia of short duration, with CNS invasion by 24 h p.i. and clearance by day 7. However, in SCID mice this virus establishes persistent viremia and infection of the CNS [7].

A similar pattern has also been seen with flaviviruses. West Nile virus (WNV) is a neuroinvasive flavivirus which causes encephalitis and death in weanling mice after i.p. or i.c. inoculation. WN-25 is a non-neuroinvasive variant, which differs from WNV in the envelope proteins and causes encephalitis and death in weanling mice only when injected i.c.[3]. While WNV showed similar patterns of invasion to the CNS in CB-17, nude and SCID mice, WN-25 could only invade the brain of SCID mice, following a prolonged high viremia [10] or when the high viral load was induced by macrophage depletion [2].

Several studies described a linkage between viremia and neuroinvasion, i.e. for Sindbis virus [15] and for Bunyavirus [14]. In contrast to the results reported here using adult mice, most studies have been done with suckling mice, in which neither the immune system nor the BBB are fully developed. One study [13] with Japanese encephalitis virus demonstrated a close relationship between virus multiplication in peripheral tissues and CNS involvement. After peripheral inoculation of the virus, two phases of virus multiplication were demonstrated: the early phase in peripheral tissues (including viremia) and the secondary phase taking place in the brain.

The unique host-virus system described here comprises four variants of Sindbis virus, which vary in their neurovirulent and neuroinvasive traits. Using this system, we have initiated a study aimed to define parameters affecting the outcome of infections caused by encephalitic viruses. In this manuscript we described and discussed experiments that emphasize the role of the host immunocompetence in neuroinvasion.

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