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Pathogenesis of murine encephalitis limited by defective interfering particles. An immunohistochemical study

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To determine whether defective interfering (DI) particles alter viral encephalitis BALB/c mice were inoculated intranasally with standard vesicular stomatitis virus (VSV) and its DI particles. Addition of 10^7 PFU equivalents of DI particles to 10^5 PFU of VSV reduced morbidity but did not delay disease onset. Less mortality was also observed. When 10^3 PFU equivalents of DI particles or UV-irradiated DI particles were substituted, these effects were absent. Attempts to correlate mortality with virus recovered from the brain could not be made due to considerable variations in the few surviving mice. Immunohistochemical analysis obtained from 121 mice showed that inoculation of DI particles limited the specific pathways of VSV antigen dissemination within the central nervous system, and new pathways were not substituted. In the group of mice with reduced mortality due to DI particles, at day 4 post inoculation VSV antigen was limited to the outer layers of the glomeruli of the olfactory bulb and to the accessory olfactory bulb, whereas there was deeper invasion of the olfactory bulb and olfactory ventricular system with mice infected with standard VSV alone. Correlation between mortality and extent of invasion became more difficult to make from 8 days on, when VSV antigens were found in discrete areas of the brain. By 12 days, few surviving mice contained any detectable VSV antigen in their brains. These results demonstrate that DI particles have potential as therapeutic agents. Also, mortality resulting from VSV-induced encephalitis, although poorly understood, may be determined very early, possibly while the virus is replicating at the site of inoculation.

Keywords: defective interfering particles; neuropathogenesis; olfactory system; immunohistochemistry; intranasal infection

Introduction

Defective interfering (DI) particles are a class of subgenomic deletion mutants that are generated by most viruses. In animal cell cultures there is ample evidence that these particles inhibit the growth of the virus from which they are derived, the so-called standard virus. Although it has long been suspected that DI particles may ameliorate viral diseases in animals, similar to the role they play in plants, proof for such a role in animals has been lacking. Moreover, some animal studies have led to unpredictable results where DI particles sometimes protect but at other times enhance the lethality of the infection (see reviews: Holland, 1987; Huang, 1988).

One of the most thoroughly studied viral systems in relation to DI particles is vesicular stomatitis virus (VSV). This is because the bullet shape permits separation of longer, replication-complement, standard virions from shorter DI particles (Huang *et al*, 1966), thus permiting constitution of viral preparation based on quantitative determinations of the two viral populations. The prototype VSV DI particle is DI-0.33 (Reichmann *et al*, 1980); it does not contain any complete genes and is, therefore, unable to express any viral proteins or to replicate by itself. Rapid and efficient replication of DI particles occurs when cells are coinfected with standard VSV as helper. Structurally, DI particles are similar

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to standard virions except that they are shorter bullet-shaped particles.

Animal studies with VSV DI particles show that inoculation leads to humoral and cellular immunity, although qualitatively less robust than that induced by infectious virus (Huang *et al*, 1966; Browning *et al*, 1991). DI particles stimulate CD4+ T lymphocytes and sensitize targets for major histocompatibility (MHC) class II recognition but interfere with MHC class I recognition by CD8+ cells (Browning *et al*, 1991). Experimental infections of animals with DI particles have yielded a variety of apparently contradicting observations. Although significant protection of mice from VSV encephalitis was reported (Cave *et al*, 1984; Forger *et al*, 1991), protection does not relate to a single ratio of standard VSV to DI particles or to the absolute quantity of DI particles. Such variable results have been attributed to the dynamic continuous and variable interaction between the two populations: standard and DI, and host defense mechanisms (Cave *et al*, 1985). Most troubling, however, has been the finding that intracerebral inoculations of increasing concentrations of DI particles resulted in greater mortality rather than greater protection (Roux *et al*, 1991). However, hamsters that are highly susceptible to intraperitoneal infection with VSV are protected by a high dose of DI particles (Fultz *et al*, 1981). Such conflicting results may have been due to different routes of inoculation, the age, strain and species of the animals, and the purity of viral preparations.

To explore how DI particles influence disease processes and what their natural roles are during viral infection, it would be necessary to localize viral distribution and spread in the absence and presence of added DI particles after inoculation through a natural route. Questions to be answered are: Do DI particles reduce the amount of standard virus in the infected animal? Do DI particles limit the spread of the virus? Do DI particles alter the routes of viral spread? Such questions can be answered using the well-defined model of murine encephalitis caused by intranasal inoculation of VSV. Considerable ground work has been done to replicate the disease symptoms (Miyoshi *et al*, 1971; Rabinowitz *et al*, 1976; Forger *et al*, 1991) and to map the routes of VSV spread in the central nervous system (Lundh *et al*, 1987; Huneycutt *et al*, 1994). In addition, the olfactory system is especially suited for such studies because nearly all processing regions are laminated, making subtle viral distributions easy to detect. The cell types have physically separate input and output pathways, and the central olfactory structures and their connections are delineated (Scott, 1985; Shepherd, 1990; Brunjes, 1994).

In this report immunocytochemical methods were used to characterize the pattern of VSV antigen distribution in the brains of BALB/c mice following intranasal inoculation with VSV and DI particles. These patterns were correlated with observed morbidity as measured by hind limb paralysis, weight loss, and reduced activity as well as mortality resulting from VSV-induced encephalitis.

Results

Effect of DI particles on morbidity and mortality caused by intranasal inoculation of mice with VSV

To determine the gross parameters of coinfection with VSV and its DI particles, several experiments were conducted where mice were individually marked, infected intranasally with VSV, and their morbidity and mortality observed for 15 days afterwards. Results from two representative experiments are shown in Figure 1. Groups of 10–12 mice were

Table 1 Effect of DI particles on the accumulation of VSV in brains of coinfected mice

Standard VSV	*DI particles*	*UV-treatment DI particles*	*Geometric mean VSV titer in brains ± s.e.m. (log 10)*		
pfu mouse^{-1}	*eq. mouse^{-1}*	*pfu eq. mouse^{-1}*	*Day post infection*		
			4	*8*	*12*
10^5			4.48 ± 0.15 (15)	4.10 ± 0.75 (3)	< 1.0 (2)
10^5	10^7		3.47 ± 0.23 (15)*	1.40 (3††*)	< 1.0 (3)
10^5	10^3		4.42 ± 0.22 (14)	2.65 ± 1.20 (3†)	nd
	10^7		3.27 ± 0.23 (15)*	5.30 ± 1.31 (4)	< 1.0 (3)
10^5		10^7	3.67 ± 0.38 (5)	4.80 ± 0.54 (4)	< 1.0 (2)
10^2			2.30 ± 0.47 (4)	4.35 ± 0.80 (4)	< 1.0 (3)
		10^7	< 1.0 (4)	< 1.0 (3)	< 1.0 (5)

A total of 114 BALB/c male mice was infected intranasally in the left nostril with standard VSV alone or together with different amounts of DI particles. Four, 8 and 12 days later mice were sacrificed and their brains were assayed for PFU on monolayers of CHO cells. Data are presented as the geometric mean titer Log10 PFU obtained from individual samples analyzed ± SEM. (*) significantly different ($P < 0.05$) from the group infected with VSV at 10^5 PFU mouse^{-1}; (nd) not determined; ({2}) one and ({3}) two mice had undetectable titers of VSV. Number of animals is given in parentheses. Data compiled from two separate experiments are presented.

inoculated with standard VSV alone at 2 concentrations (10^5 and 10^2 PFU), with DI alone at one concentration (10^7 PFU equivalents), and coinfected with both standard virus and DI particles at two relative concentrations (standard:DI at 10^5:10^7 and at 10^5:10^3). Because the DI preparation contained a 1% contamination by standard virions, the group coinfected with standard:DI at 10^5:10^7 was essentially

Table 2 Viral antigen distribution in brains of mice infected with standard VSV alone or coinfected with DI particles

Stand VSV pfu	*DI particl. pfu eq.*	*Mouse no.*	*Structures of brain*															
			Main olfactory bulb															
			GL	*EPL*	*IPL*	*IGrL*	*AOB*	*E/OV*	*AON*	*LOT*	*Tu*	*LV*	*3V*	*SC*	*Hi*	*T*	*Hy*	*VTA*	*BrS*
***(a)** 4 days post infection*																			
10^5		1	+++	+++	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-
		2	+++	+++	+++	+++	++	-	+	-	-	-	-	-	-	-	-	-	-
		3	+++	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
10^5	10^7	1	++	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
		2	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
		3	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
10^5	10^3	1	+++	+++	++	++	+	+++	++	-	-	-	-	-	-	-	-	-	-
		2	++	-	-	-	++	+	-	-	-	-	-	-	-	-	-	-	-
		3	+++	++	+	-	++	-	-	-	-	-	-	-	-	-	-	-	-
	10^7	1	++	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
		2	++	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
		3	+	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
10^5	10^{7*}	1	++	+++	++	++	++	+++	++	-	-	-	-	-	-	-	-	-	-
		2	+++	++	++	++	+++	-	-	-	-	-	-	-	-	-	-	-	-
		3	++	++	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
10^2		1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10^{7*}	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
***(b)** 8 days post infection*																			
10^5		1	++	+	+	+	++	+	++	-	+++	+	++	++	+	-	+	+	-
		2	++	+	+	-	++	-	+	-	-	-	+	-	-	-	+	+	++
		3	+	++	+	++	-	+	-	-	-	-	+	-	-	-	+	++	++
10^5	10^7	1	++	+	+	+	++	-	-	-	-	-	-	-	-	-	-	-	-
		2	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
		3	++	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
10^5	10^3	1	++	+	-	-	++	-	+	-	-	-	-	-	-	-	-	-	-
		2	++	++	++	++	++	-	+	-	-	+	+	+	-	-	+	-	++
		3	++	++	++	+	++	-	+	-	++	+	+	++	+	-	+	-	+
	10^7	1	++	-	-	-	++	+	-	-	+	+	+	+	-	-	++	-	-
		2	++	-	-	-	++	-	+	-	+	+	++	-	-	+	+	+	-
		3	++	-	-	-	++	-	-	-	-	-	-	+	-	-	+	-	-
10^5	10^{7*}	1	++	-	-	-	++	-	++	-	-	-	-	+	+	-	+	-	-
		2	++	-	-	-	++	-	+	-	-	-	+	-	+	-	++	-	-
		3	++	-	-	-	++	-	-	-	-	-	-	-	-	-	+	-	-
10^2		1	++	-	-	-	+	+	-	-	-	+	+	+	-	+	++	-	-
		2	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10^{7*}	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2 Continued

Stand VSV pfu	DI particl. pfu eq.	Mouse no.	Structures of brain: Main olfactory bulb GL	EPL	IPL	IGrL	AOB	E/OV	AON	LOT	Tu	LV	3V	SC	Hi	T	Hy	VTA	BrS
(c) 12 days post infection																			
10^5		1	+	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	+
		2	+	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
		3	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
10^5	10^7	1	+	+	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
		2	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
		3	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
10^5	10^3	1	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
		2	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
		3	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	10^7	1	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
		2	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
		3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10^5	10^{7*}	1	+	-	-	-	+	-	-	-	-	-		-	-	-	-	-	-
		2	+	-	-	-	+	-	-	-	-	-		-	-	-	-	-	-
		3	+	-	-	-	+	-	-	-	-	-		-	-	-	-	-	-
10^2		1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	++	-	-	-	-	-	-	-	-	-	++	++	-	-	++	-	-
		3	+	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
	10^{7*}	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

A total of 63 BALB/c male mice divided into groups of seven were infected intranasally in the left nostril with VSV alone or with combinations of VSV and DI particles. Three mice from each group were sacrificed at 4, 8 and 12 days post infection and perfused; the left side of their brains were sectioned sagittally and stained for VSV antigen. Twenty to 30 sections from each animal were examined. The presence of antigenic material was scored: (-) negative; (+) slight; (++) moderate and (+++) intense staining of sections; (*) UV-treated.

identical to the group infected with just 10^7 PFU equivalents of DI particles.

Weight loss, as measured by a difference of 10% or greater, was evident in some of the mice as early as 4 days after inoculation (Figure 1a). Mice infected with standard VSV alone showed a reproducible pattern of weight loss, with the maximum number in each group affected between 7–8 days after infection. Fewer animals showed weight loss when they were infected with the lower dose of VSV. When the higher dose of 10^7 PFU equivalents of DI particles was mixed with the higher dose of standard VSV (10^5 PFU) to coinfect mice there was, also, a reduction in the number of mice showing weight loss without significantly showing a delay in the time of its onset, suggesting that DI particles were able to reduce morbidity but not delay it. However, when the concentration of DI particles was reduced to 10^3 PFU equivalents the protective effect was lost. DI particles alone at 10^7 PFU equivalents led to the same weight loss pattern as mice coinfected at the high doses of standard virus and DI particles. Because this preparation of DI particles contained contaminating standard virus up to a level of 10^5 PFU, the pattern and number of animals exhibiting weight loss was similar to mice coinfected at the highest concentrations. In contrast, when DI particles were absent and mice were infected at a much lower concentration of VSV alone (10^2 PFU) there were more mice showing weight loss, suggesting, again, that DI particles were essential for moderating the infection.

Hind limb paralysis (Figure 1b) among these same groups of mice also peaked at 7–8 days post inoculation. Overall fewer mice exhibited hind limb paralysis than weight loss with the groups falling into two distinct patterns. Those groups infected with standard virus alone, at either the high or low doses, and those coinfected with standard virus and a low dose of DI particles had about 50% paralyzed in at least one leg, demonstrating that significant morbidity was caused by standard virus alone at either concentration or when the effect of DI particles was diluted out. The second pattern of hind limb paralysis had a lower percentage of the mice affected, showing that DI particles matched against 10^5 standard virus reduced paralysis. Along with this reduction, however, a few animals infected

with the high concentration of DI particles continued to exhibit paralysis and/or weight loss up to the 13th day, whereas mice from the more severely affected groups either died by day 10–11 or survived. Prolonged paralysis may be indicative of residual effects caused by DI particles, but did not persist beyond the 14th day. In general, there was a high degree of correlation between hind limb paralysis and mortality (Plakhov, unpublished observations). Ruffled dull fur and reduced activity were also noted. These symptoms correlated with the other parameters except that they preceded paralysis and weight loss (data not shown).

Control groups of mice (data not shown), inoculated at the same time as these groups with UV-treated DI particles at 10^7 PFU equivalents, or mock infected, did not show any morbidity throughout the observation period. Also, when mice were coinfected with the high concentration of standard VSV and UV-irradiated DI particles up to 10^7 PFU equivalents, none of the ameliorating effects of DI particles was seen.

In three separate experiments mortality was observed to track with the other disease parameters. A representative experiment, different from that shown in Figure 1a and b is shown in Figure 1c. Mice infected by standard virus alone at 10^5 or 10^2 PFU or with diluted DI particles sustained a 60% mortality rate that occurred between day 7–10 after infection. Those infected with the DI preparation alone or coinfected with the higher concentrations of VSV and DI particles (10^5:10^7, respectively) showed reduced mortality, suggesting that DI particles were protective. Control groups of mice (data not shown) coinfected with VSV at 10^5 PFU and UV-irradiated DI particles up to 10^7 PFU equivalents had the same high mortality as those infected with standard VSV alone. Irrespective of the inoculum, mortality occurred at about the same time for all the groups, suggesting that there was a brief window of time for determining the outcome before specific host defenses became effective. Infection of mice with UV-treated DI particles alone did not lead to death in any of the mice.

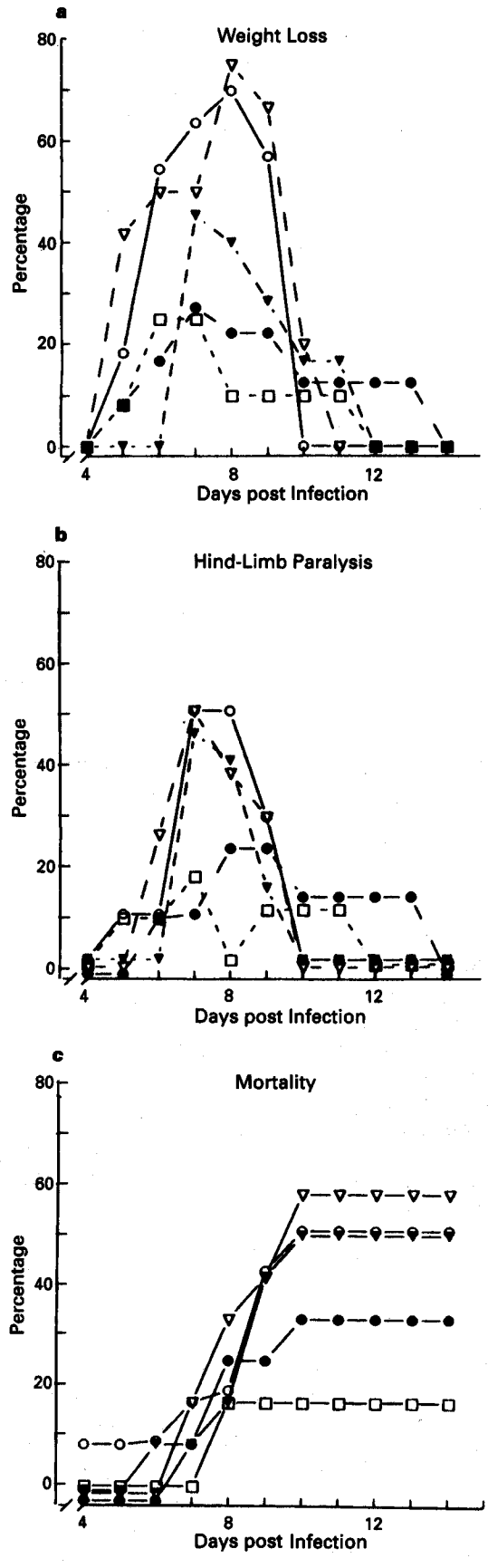

Figure 1 Effect of coinfection with standard VSV and DI particles on weight loss, hind-limb paralysis, and death in mice. During a 14 day period of observation weight loss (a); hind limb paralysis (b); and deaths (c) were recorded. Groups of 12 male BALB/c mice were infected intranasally in their left nostril with standard VSV alone at a dose of 10^5 PFU mouse^{-1} (open circle) or 10^2 PFU mouse^{-1} (closed inverted triangle), standard VSV administered with DI particles: standard VSV at 10^5 PFU mouse^{-1} and DI particles at 10^7 PFU eq. mouse^{-1} (closed circle), standard VSV 10^5 PFU mouse^{-1} and DI particles 10^3 PFU eq. mouse^{-1} (open inverted triangle), and DI particles alone at 10^7 PFU eq. mouse^{-1} (open square). Representative data from one out of three experiments are presented.

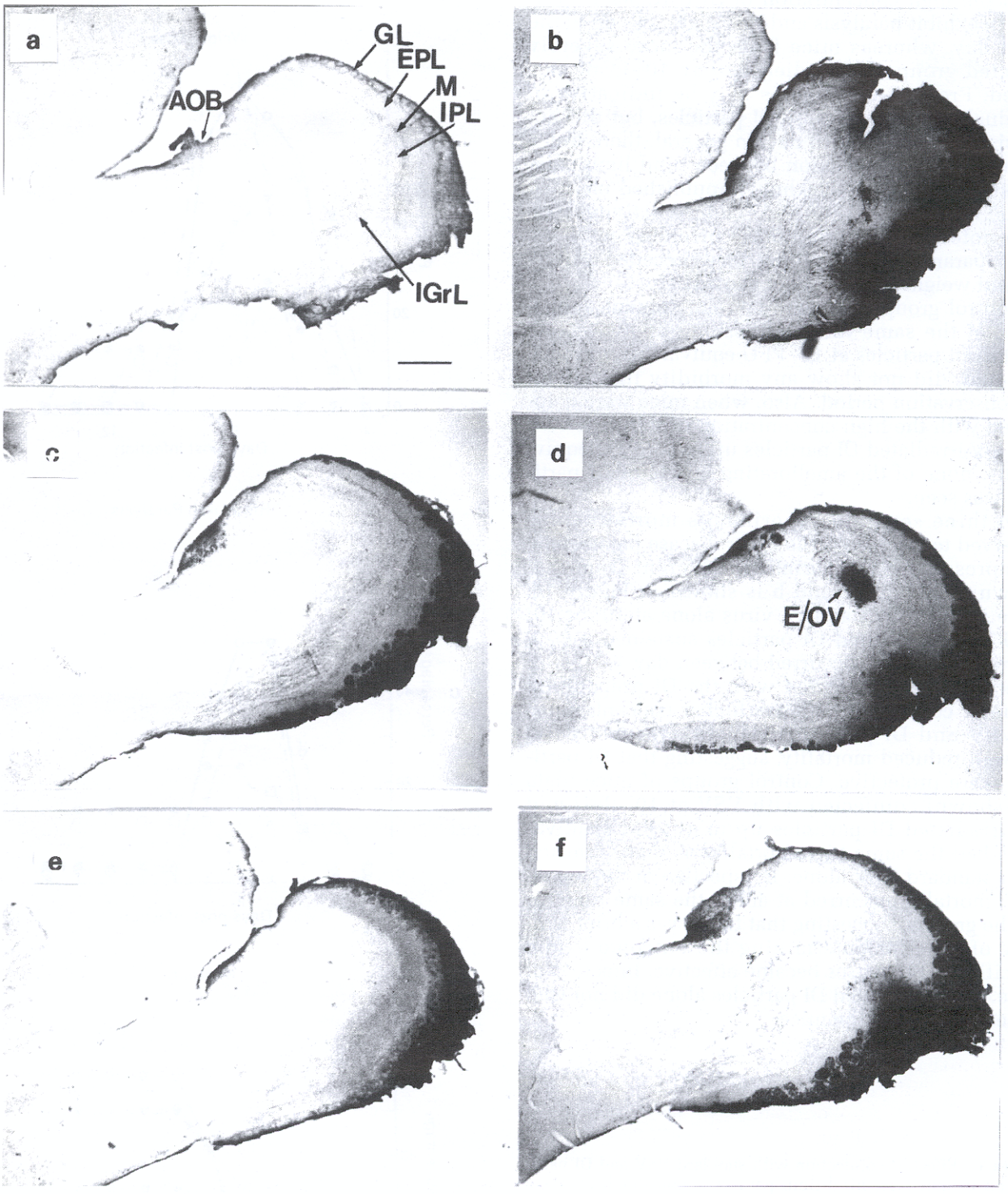

Figure 2 Immunohistochemical staining of the olfactory bulb of mice infected intranasally with standard VSV with or without DI particles at 4 days post infection. Mice at 4 days post inoculation in the left nostril were perfused and the left half of their brains prepared for sagittal sectioning. (a) uninfected mouse. No specific labeling was observed within this brain tissue. (b) infected with standard VSV at 10^5 PFU. VSV antigens were present along ventral and dorsal surfaces of the main olfactory bulb. Immunoreactivity is present within the GL; EPL; IPL; IGrL, and the AOB. (c) coinfected with standard VSV at 10^5 PFU and DI particles at 10^7 PFU eq. Immunoreactivity was present only in GL and AOB. (d) coinfected with standard VSV at 10^5 PFU and DI particles at 10^3 PFU eq. Intense VSV immunoreactivity was present in the E/OV. (e) infected with DI particles at 10^7 PFU eq. Specific immunoreactivity was present in GL and AOB. (f) coinfected with standard VSV at 10^5 PFU and UV-treated DI particles at 10^7 PFU eq. VSV immunoreactivity was intense in the GL; EPL, and IPL. Bar = 0.5 mm for all panels. *Abbreviations:* GL, glomerular layer; EPL, external plexiform layer; IPL, internal plexiform layer; IGrL, internal granular layer; AOB, accessory olfactory bulb; E/OV, subependymal layer surrounding the olfactory ventricle; AON, anterior olfectory nucleus; LOT, lateral olfactory tract; Tu, olfactory tubercle; LV, lateral ventricle; 3V, third ventricle; SC, septal complex; Hi, hippocampal formation; T, Thalamus; Hy, hypothalamus; VTA, ventral tegmental area; BrS, brainstem.

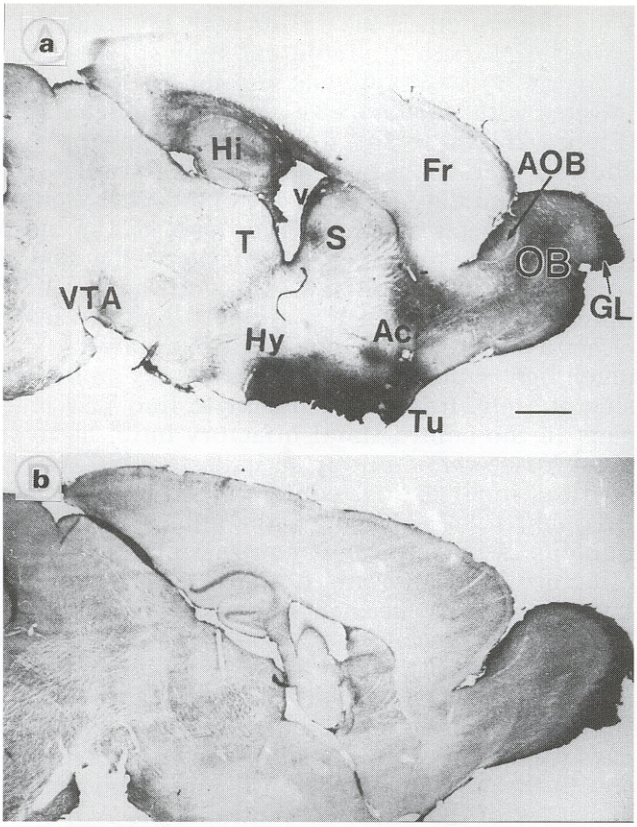

Figure 3 Immunohistochemical staining of the brain of mice infected intranasally with standard VSV with or without DI particles after 8 days. (a) shows a sagittal section of the left side of the brain of a mouse infected with standard VSV at 10^5 PFU. Note the immunoreactivity extending from the olfactory bulb (OB) to caudal regions: Fr, frontal lobe of cerebral cortex; Ac, nucleus accumbens; Tu; Hy; S, striatum; T; Hi; V, lateral ventricle; VTA. (b) shows a sagittal section of the left side of the brain of a mouse infected with standard VSV at 10^5 PFU together with DI particles at 10^7 PFU eq. VSV-immunoreactivity was observed only in the olfactory bulb: GL and AOB. Bar = 1 mm for all panels.

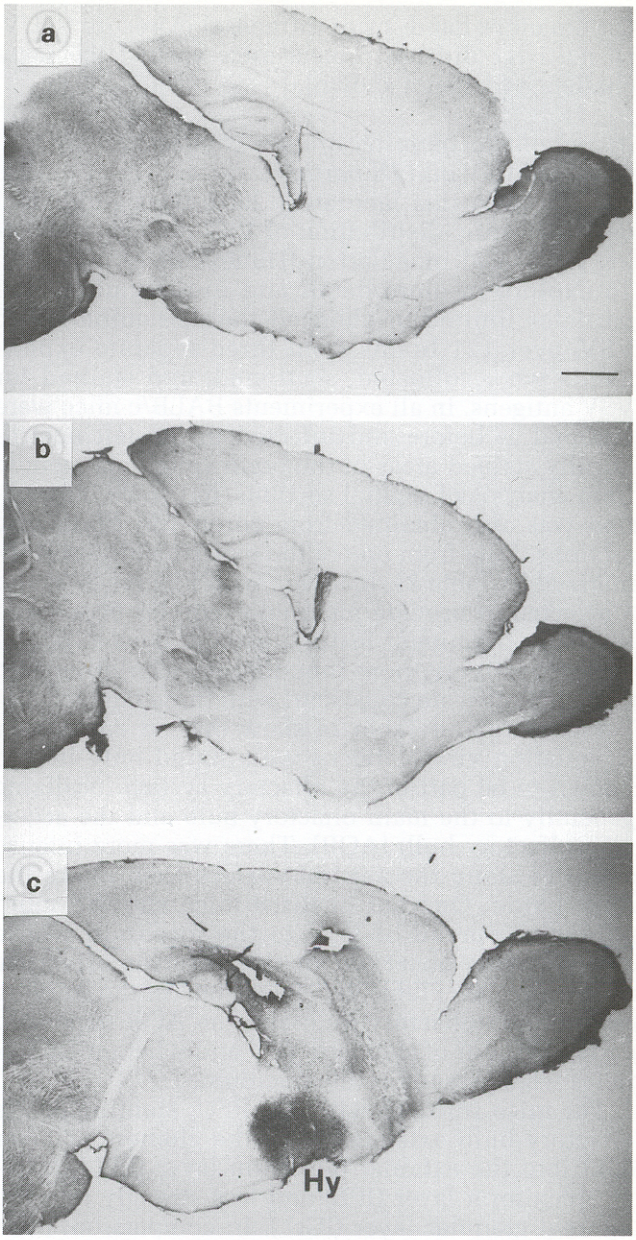

Figure 4 Immunohistochemical staining of the brain of mice infected intranasally with standard VSV with or without DI particles after 12 days. (a) shows a sagittal section from a mouse infected with standard VSV at 10^5 PFU and (b) shows a sagittal section from a mouse coinfected with standard VSV at 10^5 PFU and DI particles at 10^7 PFU eq. There is residual VSV-immunoreactivity only in the GL of the olfactory bulb and in the AOB in both cases. (c) shows a sagittal section from a mouse infected with the low dose of standard VSV at 10^2 PFU: VSV is present in the olfactory bulb and in more caudal regions of brain such as the Hy. Bar = 1 mm for all panels.

Recovery of VSV from brains of mice coinfected with standard VSV and DI particles

To determine whether morbidity and mortality correlated with virus load in the brain, brain homogenates from infected BALB/c mice and control groups were obtained on 4, 8, and 12 days following inoculation and the plaque-forming virus content titered. At 4 days all mice, which received standard virus, had recoverable PFU (Table 1). At 4 days, mice infected with low doses of VSV alone or together with DI particles, had less virus than those infected with the higher dose of standard VSV alone. This pattern became less consistent at 8 days post inoculation; and by day 12 infectious virus was undetectable in surviving mice.

Mice infected with UV-irradiated DI particles alone failed to yield any infectious virus. Also, mice surviving the coinfection with standard VSV and DI particles were observed for 30 days for any evidence of persisting virus in the brain. None was detected. Upon subsequent challenge with 10^6 PFU of VSV on day 30, all mice infected either with VSV or DI particles alone or coinfected with VSV and DI particles were protected and free from any sequelae.

On the other hand, prior intranasal exposure to UV-DI particles did not protect mice against the same challenge (data not shown).

Immunohistochemistry of mice coinfected with standard VSV and DI particles

Studies from this laboratory have been published on the specific distribution of VSV antigens following intranasal inoculation (Huneycutt *et al*, 1994). To explore in more detail and to determine if DI particles limited or changed the dissemination of VSV, over 121 brains from three separate experiments were collected, sectioned, and stained for VSV antigens. In all experiments BALB/c mice were grouped as before and infected in the left nostril. Locally stained areas of the brain were documented from analysis of sagittal sections, but some information was also obtained from coronal or horizontal sections. Data from one of the experiments are summarized in Table 2a, b, and c, with Figures 2, 3, and 4 selected to provide pictorial examples of some of the more prominent features seen at days 4, 8, and 12, respectively, post inoculation. The other two experiments were consistent with these data.

Figure 2a and Table 2a show that on day 4 after inoculation viral antigens were found only in loci known to be part of the olfactory system localizing generally to the main olfactory bulb and the accessory olfactory bulb (AOB). There was more intense staining along the ventral surface than along the dorsal side. One out of three mice showed more extensive spread of VSV to the forebrain where staining was observed in the anterior olfactory nucleus (AON) (Table 2a). The extent of the staining in the main olfactory bulb and the AOB depended on the amount of standard VSV and whether DI particles were present in the inoculum.

For mice infected with 10^5 PFU, staining of the olfactory bulb extended from the glomerular layer (GL) into the external plexiform layer (EPL), internal plexiform layer (IPL) and less strongly into the internal granular layer (IGrL). In comparison, mice infected with the lower dose of standard virus (10^2 PFU) alone showed much less staining overall in the olfactory bulb; the tissue that was stained was mainly the outer glomerular layer (Table 2a). Addition of 10^7 PFU equivalents of DI particles to the higher dose of standard virus reduced staining in the internal layers of the main olfactory bulb but the outer glomerular layer remained strongly stained (Figure 2c; Table 2a). A similar limited pattern of distribution of VSV antigens was observed at 4 days in the group of mice infected with the DI preparation containing 10^7 PFU equivalence, but containing 10^5 contaminating standard VSV (Table 2a). Also, mice infected with the low dose of standard VSV showed evidence of VSV antigens only in the glomerular layer of the ipsilateral olfactory bulb (Table 2a). Mice infected with standard VSV at 10^5 PFU together with DI particles at 10^3 PFU equivalents (Figure 2d; Table 2a) or with UV-treated DI particles (Figure 2f; Table 2a) showed extensive invasion by VSV antigens similar to those mice infected with standard VSV alone at the higher titer (Figure 2b; Table 2a). Both of these groups also showed more immunoreactivity in the olfactory ventricle and the AON than any of the other groups.

Sagittal sections of the right side of the brain (data not shown), which is opposite to the side of intranasal inoculation, showed some VSV immunoreactivity in the glomeruli of the main olfactory bulb and in the AOB, but staining was much lighter than in the same structures on the left side of brain. It is not surprising to find VSV antigens in the contralateral olfactory bulb, because nasal fossae communicate and olfactory bulbs have bi-directional axonal connections (Shipley and Adamek, 1984). Moreover, the anterior olfactory nucleus is an important contralateral source of olfactory bulb afferents (Carson, 1984) and it is known that VSV uses anterograde and retrograde transport (Huneycutt *et al*, 1994). At 4 days sagittal sections showed that neither side of the forebrain, midbrain and hindbrain areas was infected. However, upon coronal sectioning a few mice at 4 days were observed to contain viral antigens ipsilateral to the VSV instilled nostril in the subependymal layer of the olfactory ventricle, the AON, the septal complex, the caudate-putamen nucleus, the nucleus horizontal limb diagonal band, the nucleus vertical limb diagonal band, and the lateral hypothalamic area. On the other hand, the lateral olfactory tract, lateral and third ventricles were antigen negative. In mice inoculated with UV-treated DI particles alone, viral antigens were not detected anywhere in the central nervous system on days 4, 8 or 12 post inoculation (Table 2a,b,c).

By 8 days post inoculation with only standard VSV at 10^5 PFU, staining extended into the ependymal layer of the olfactory ventricle, the lateral and third ventricles, the septal complex, the hypothalamus, the hippocampus, the ventral tegmental area, and the brainstem (Figure 3a; Table 2b). However, coinfection with the protective dose of DI particles led to limited antigen dissemination. Within the main olfactory bulb and the AOB, staining remained the same or increased slightly from day 4, but never reached the same extent or intensity as in mice infected with 10^5 PFU of standard virus alone (Figure 3b). In one out of three mice in this group VSV immunoreactivity extended into the EPL, the IPL and the IGrL (Table 2b). Invasion of viral antigens from the olfactory bulb into the caudal regions of the brain was distinct with one group of mice (10^5 PFU VSV and 10^7 PFU equivalents of DI particles) showing virtually no antigen in the more caudal regions. In contrast in the other group, infected with only 10^7 PFU equivalents of DI particles, VSV antigens were observed in various areas of the forebrain, midbrain and hindbrain (Table 2b). The pat-

tern of VSV spread in the brains from mice infected with 10^5 PFU of standard VSV together with 10^3 PFU equivalent of DI particles or with 10^7 PFU equivalent of UV-DI particles was very similar to mice infected with only standard VSV at 10^5 PFU (Table 2b). In one other group, one of three mice infected with the low dose of standard VSV showed immunoreactivity extending from the olfactory bulb into caudal regions of the brain (Table 2b).

By 12 days post inoculation the majority of the surviving animals in all groups had reduced viral antigen staining, limited to the glomeruli of the main olfactory bulb and the AOB; the infection appeared to resolve since the staining appeared lighter than at 4 days post inoculation (Figure 4a,b; Table 2c). Mice inoculated with the much lower titer of 10^2 PFU of standard VSV had a pattern of VSV antigen dissemination that was greater than that of brains infected with 10^5 PFU. Still, the degree of infection was less than expected based on the mortality rate. On each of the observation days, antigen was detected only in small amounts in the outer glomerular layer of the olfactory bulb and in the AOB. By day 12, when caudal regions of the brain were no longer stained in most of the surviving mice from the other groups, this group still had significant, but variable staining (Figure 4b; Table 2c). The discrepancy between the high mortality observed, as well as the high virus titers found in the brain at 8 days post infection and the limited antigen found on histochemical staining, suggest that mortality may not be related to the extent of virus spread within the olfactory bulb.

Discussion

These results demonstrate that VSV DI particles reproducibly reduced morbidity and mortality when inoculated intranasally into mice at the same time with standard VSV. This reduction was accompanied by limited viral dissemination into the olfactory bulb. Reproducibility was dependent on the quantitative characterization of DI preparations in terms of contaminating standard VSV and controlling the volume of the inoculum and its method of delivery.

Examination of immunohistochemical sections of over 121 mice indicates that the correlation between mortality and the extent of viral antigen distribution, seen in the layers of the olfactory bulb and the olfactory ventricle of the brain, was evident by 4 days post inoculation. By day 8, however, such correlations were difficult to make because viral antigen distribution became more extensive, disseminating from the ventricular regions into the forebrain, midbrain, and hindbrain. By 12 days post inoculation, viral antigen was again more limited, this time to the outer glomerular layer of the olfactory bulb and to the AOB. Subsequent examination of survivors did not show any residual infectious virus nor evidence of viral antigens (data not shown). This contrasts with the observations of Fultz and Holland, 1984, where viral persistence was observed in hamsters, as evidenced by recovery of VSV from brain, spleen and liver homogenates as long as 8 months post infection and demonstration of viral antigens in the liver, kidney, and brain 4 to 16 months post infection.

One group of mice did not fit the general trend. Those infected with 10^2 PFU of standard VSV had the same high mortality rate as those inoculated with 10^5 PFU and yet at 4 days there was very little viral antigen in their olfactory bulbs. This discrepancy needs further study, but highlights the finding that involvement of the internal layers of the olfactory bulb as well as the forebrain, midbrain, and hindbrain beyond the olfactory bulb may be unrelated to mortality.

Similarly, it is difficult to assign any real significance to the increase or decrease in the geometric mean VSV titers on days 4 and 8. The titer of VSV did not correlate significantly with the rate of survival for their group. Nonetheless, from these results and from many similar observations (Cave *et al*, 1985; Forger *et al*, 1991) evidently virus replication in mouse brains precedes by several days the development of symptoms such as weight loss, hind limb paralysis, and mortality. Although there may be a trend for delayed virus replication in the CNS and less overall recoverable virus among the groups with lower morbidity and mortality, it must be remembered, as well as for the histochemical studies, that viral titers, especially from mice inoculated 8 days or more previously, were only determined from the survivors who may not be representative of their group.

The findings that factor(s) other than recoverable virus from the brain may contribute to mortality and that spread of VSV beyond the glomerular layer of the olfactory bulb may be unrelated to mortality suggest that determinants of whether a mouse dies or not are not well understood. The outcome may have been determined very early after intranasal inoculation where VSV is thought to replicate prior to viral entry into the CNS. Olfactory sensory epithelium, as has been suggested in analogy with the blood–brain barrier, prevents exogenous environmental substances from reaching the central nervous system (Baker, 1988). That brains of mice inoculated with UV-treated DI particles alone did not show any staining for VSV antigens, indicates that entry of VSV into the central nervous system through this barrier requires multiplication of VSV. The crucial site may be deduced from the consistently intense staining of the glomeruli in the main olfactory bulb and accessory olfactory bulb. Axons of olfactory sensory neurons, residing in the nasal epithelium, pass out of this region through small openings at the base of the skull (cribriform plate), and connect to the glomerular layer of the olfactory

bulb. The accessory olfactory bulb, on the other hand, connects with neurons from the vomeronasal organ. VSV, like rabies virus (Lafay *et al*, 1991), infects the neuroepithelia in the olfactory mucosa and the vomeronasal organ but not the respiratory epithelium in the nasal cavity (Lundh *et al*, 1987). Although it is not clear how virus gets from the nasal epithelium to the olfactory bulb, it is unlikely to be through a non-specific route via the ependymal surfaces because VSV antigen appeared in layers of cells in the olfactory bulb and was limited to those neurons that form part of the olfactory system. Therefore, successful intervention strategies need to be focused on viral entry into the central nervous system or to reduce viral replication at the initial point of entry.

Because very little is known about the cause(s) of mortality in VSV-infected mice, efforts, also, need to be focused on identifying how VSV causes murine death. It was commonly assumed that hind limb paralysis led to dehydration and death from an inability to reach water. This is now known not to be true. Mortality may be the result of destructive immune responses or direct lytic effects of the virus on critical centers of the CNS or brainstem, which have not yet been identified, that control essential functions such as respiration or blood flow.

VSV antigen distribution, especially early after intranasal inoculation, is surprisingly specific to areas of the brain known to be involved with the olfactory system (Lundh *et al*, 1987; Lundh *et al*, 1988; Huneycutt *et al*, 1994) despite the fact that *in vitro* VSV infects virtually every cell type. It is thought that VSV exits from polarized epithelial supporting cells and olfactory neurons through the basolateral route, but not from their apical surface or through the ciliated bulbous endings of the olfactory neuronal dendrites (Rodriguez-Boulan and Sabatini, 1978; Fuller *et al*, 1984; Rigaut *et al*, 1991). The findings reported here are consistent with previous publications that VSV spread along the olfactory nerves into the glomeruli of the olfactory bulb and then into other areas of the brain by using olfactory axonal pathways (Lundh *et al*, 1987; Andersson *et al*, 1993; Huneycutt *et al*, 1994). Such specificity places VSV among a handful of viruses that have been shown to be useful for neurosynaptic tracing when inoculated through a natural route. How such directionality is determined and what prevents general centrifugal spread throughout the brain remains unknown. In contrast, when VSV is inoculated intracerebrally there is generalized spread of the virus into the damaged areas (Sabin and Olitsky, 1938). Also, when the olfactory bulb was surgically ablated intranasal inoculation led to widespread antigen dissemination especially along the damaged surfaces of the brain (Plakhov *et al*, submitted).

These studies demonstrate that DI particles reproducibly reduced mortality and limited the spread of viral antigens into the olfactory bulb if inoculated intranasally at the same time as standard VSV. The routes of spread within the CNS were not altered in the presence of DI particles. Unfortunately, it was not possible to determine with any accuracy whether DI particles reduced the amount of virus in the CNS. Nevertheless, DI particles remain as potentially therapeutic agents for manipulating the outcome of viral diseases in animals.

Materials and methods

Standard virus and DI particles

Clonally purified VSV of the Indiana serotype, San Juan strain, was propagated in Chinese hamster ovary (CHO) cells and purified by rate zonal centrifugation in sucrose gradients (Huang and Manders, 1972). Aliquots of 10^8 PFU of standard virus were stored at –70°C and used only once after freeze-thawing. Preparations of DI particles were obtained by coinfecting CHO cells with clonally purified standard VSV at a multiplicity of infection of 0.03–0.09 and purified DI stock at a multiplicity of infection of about 0.004. The resulting progeny was purified by rate zonal centrifugation (Huang and Manders, 1972). Fractions containing DI particles were pooled and subjected to a second round of centrifugation, after which the DI preparations contained less than 1% standard virus as contaminants. The concentration of DI particles was estimated from their OD_{260}. The PFU equivalent titer of DI particles was determined by assaying their ability to inhibit cumulative VSV RNA synthesis as measured in a pilot assay (Huang *et al*, 1970). In brief, CHO cells at 4×10^6 in 1 ml were mixed with actinomycin D (5 μg ml^{-1}) and VSV at a multiplicity of 10; DI particles were added at different concentrations, usually at estimated multiplicities from 20 to 0.01. The functional titer of DI preparations was determined from the lowest concentration of DI stock that inhibited VSV RNA synthesis maximally. This dilution of the DI preparation was considered to have an equal multiplicity of infection of 10 as the standard virus used for the assay.

UV irradiation of DI particles

Inactivation was carried out on 0.5 ml of purified DI particles placed in 35 × 10 mm tissue culture plates (Falcon, Becton Dickinson) which were placed at a distance of 5 cm from a germicidal UV lamp (G8T5 8w, Sylvania Electric). Exposure was for 2 min at 4°C with constant agitation. Titration of UV-treated DI preparations showed that residual interference as well as standard virus was undetectable.

Mice and their infection with VSV

Male BALB/c mice purchased from Taconic Farms Inc, Germantown, NY, were used at 5–7 weeks of age. Animals were housed five per cage and fed *ad*

libitum. Infection with VSV was conducted by lightly anesthetizing each mouse for 1 min in a closed container with methoxylfurane (Metofane™, Pitman-Moore Inc, Mundelein, IL, USA) as previously described (Forger *et al*, 1991; Huneycutt *et al*, 1994). Each animal was infected by intranasal instillation of standard VSV or DI particles alone or mixed in a total volume of 0.005 ml in the left nostril. Animals mock infected with buffer were used as negative controls. At this volume virus remained in the nostril and did not enter the bronchi. Morbidity was determined by observing the animals daily, noting their activity level, weighing them, and determining paralysis in their hind limbs.

Determination of viral titers from mouse brains

Mice were given a lethal overdose of 5 mg sodium pentobarbital (Nembutal™, Abbott Laboratories, North Chicago, IL, USA), and their brains were aseptically removed (Forger *et al*, 1991; Huneycutt *et al*, 1994), weighed, and made 10% (w/v) in cold phosphate-buffered saline with EDTA. Individual brains were homogenized with Potter-Elvejham homogenizers under standardized conditions and centrifuged at 2060 × g for 10 min. The supernatants were aliquoted and stored at –70°C before they were plaque assayed on CHO cells (Stampfer *et al*, 1969). Positive (virus of known titer) and negative (no virus) controls were included in each plaque assay; positive controls did not vary significantly from one assay to another (range, 8.7 to 9.2 log PFU ml^{-1}).

Immunohistochemistry

On selected days after virus inoculation, mice were given a lethal overdose of sodium pentobarbital and immediately perfused transcardially with 20–30 ml normal saline followed with 30–50 ml freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Whole brains were removed and post-fixed in the same paraformaldehyde solution at 4°C for less than 48 h. Twenty to thirty 40 μm sections from each brain were prepared in either the sagittal, coronal or horizontal orientation on a vibratome. Free-floating sections were stained as previously described (Huneycutt *et al*, 1993; 1994). Reagents utilized were sheep hyperimmune serum, which was generously provided by Dr Edward J. O'Rourke (The Children's Hospital, Boston) and recognizes all VSV viral proteins (Reiss *et al*, 1986; Huneycutt *et al*, 1994); biotinylated donkey anti-sheep polyclonal antibodies specific for IgG heavy and light chains (Jackson Immunoresearch Inc, West Grove, PA, USA), which detect bound sheep hyperimmune serum; avidin-biotinylated horseradish peroxidase, which amplifies the complex (Elite standard kit, Vector Laboratories, Burlingame, CA, USA); and, finally, 3,3′-diaminobenzidine (Aldrich Chemicals, Milwaukee, WI, USA), which together with H_2O_2 develops a color reaction. Rinsed sections were mounted on 2× subbed slides, dehydrated, mounted, then cover slipped using Permount. Slides were analyzed under a light microscope and photographs were made (Huneycutt *et al*, 1993; 1994).

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