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Subclinical Infections in Mice Resulting from the Modulation of a Lethal Dose of Semliki Forest Virus with Defective Interfering Viruses: Neurochemical Abnormalities in the Central Nervous System

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SUMMARY

The lethal encephalitis caused in mice by Semliki Forest virus (SFV) is modulated to a subclinical infection by administration of defective interfering SFV, although virus still multiplies both in the central nervous system (CNS) and systemically. Here we report that such infections result in unique and selective changes in the normal levels of CNS neurotransmitters some of which persist after infectious virus can no longer be detected. This represents a previously undocumented category of infection which may have a bearing on the aetiology of those human neurological and neuropsychiatric diseases to which viruses are believed to contribute.

Huang & Baltimore (1970) suggested that defective interfering (DI) viruses played a role in the expression of virus disease by attenuating infection or by converting an acute into a persistent infection. DI viruses are naturally occurring deletion mutants, often with the majority of the genome deleted, which depend on infectious (standard) virus for their replication and have the ability to interfere with the multiplication of infectious virus at the molecular level (Perrault, 1981; Dimmock, 1985). However, evidence that this occurs in infections of animals, even in model systems, has been slow to accumulate (Holland & Doyle, 1973; Spandidos & Graham, 1976; Welsh *et al.*, 1977; Dimmock & Kennedy, 1978; Jones & Holland, 1980; Fultz *et al.*, 1982; Barrett & Dimmock, 1986) and there is no evidence that DI viruses are implicated in virus diseases of man. Our recent studies have demonstrated that the normally lethal encephalitis caused in adult mice by Semliki Forest virus (SFV) could be completely prevented by treatment with DI SFV (Dimmock & Kennedy, 1978; Crouch *et al.*, 1982; Barrett & Dimmock, 1984*b*, *c*, *d*; Barrett *et al.*, 1984*b*) although virus still multiplied in the central nervous system (CNS) and elsewhere. Despite the lack of clinical signs of disease we suspected that brain function might be affected. Thus we monitored levels of the four major neurotransmitters: dopamine, 5-hydroxytryptamine, acetylcholine and γ-aminobutyric acid and in this report we describe major, selective changes which occur during this silent infection.

The virulent strains of SFV are neurotropic and multiply to high titre in the CNS where they infect and damage neurons predominantly (Crouch *et al.*, 1982; Barrett *et al.*, 1984*b*). In the current series of experiments (for methods etc., see Dimmock & Kennedy, 1978; Barrett & Dimmock, 1984*b*), 5-week-old random-bred male mice (CFLP; Hacking and Churchill, Wyton, Huntingdon, U.K.) were inoculated with DI virus 2 h before and together with 10 LD_{50} of a virulent strain of SFV (derived from ts^+; Tan *et al.*, 1969) by intranasal inoculation under ether anaesthesia. Control mice received 10 LD_{50} and, in place of DI virus, an equivalent amount of non-infectious SFV antigen (u.v.-irradiated virus) to control for possible immunogenic effects.

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Table 1. *Summary of neurochemical data*

		Number of animals	HVA (ng/mg)	5HIAA (ng/mg)	CAT (pmol/mg/h)	GAD (nmol/mg/h)
Day 4*	Mock-infected	10	1·6†	3·0	20·6	50·9
			(0·07)	(0·22)	(1·99)	(1·61)
	Virulent SFV	5	5·3‡	4·8‡	27·3	68·1
			(0·87)	(0·43)	(1·58)	(7·86)
	SFV + DI p4	20	3·5‡	3·1	24·8	37·5‡
			(0·63)	(0·30)	(1·73)	(4·42)
	SFV + DI p13a	20	5·2‡	2·8	24·7	47·1
			(0·90)	(0·25)	(1·39)	(3·60)
	Avirulent SFV	6	5·1‡	3·0	29·9	64·1
			(1·13)	(0·39)	(2·58)	(3·95)
Day 12	Mock-infected	10	1·4	2·4	27·3	48·7
			(0·12)	(0·29)	(2·91)	(4·77)
	SFV + DI p4	12	1·4	1·6	24·1	26·4‡‡
			(0·15)	(0·19)	(2·06)	(7·23)
	SFV + DI p13a	14	1·4	1·6	28·5	17·9‡‡
			(0·17)	(0·12)	(2·42)	(5·15)
	Avirulent SFV	6	1·3	2·5	30·8	48·1
			(0·08)	(0·13)	(2·53)	(7·63)
Day 21	Mock-infected	7	1·5	3·2	21·2	50·4
			(0·13)	(0·22)	(1·36)	(4·72)
	SFV + DI p4	8	1·5	3·1	19·2	50·7
			(0·21)	(0·27)	(1·26)	(5·4)
	SFV + DI p13a	13	1·8	3·2	19·4	54·7
			(0·34)	(0·15)	(1·45)	(4·3)
	Avirulent SFV	5	1·7	2·6	19·7	59·3
			(1·17)	(0·18)	(1·8)	(6·7)

* Mice were infected by the intranasal route (Dimmock & Kennedy, 1978) with either virulent SFV (10 LD_{50} = 6 × 10^3 p.f.u.), DI virus + 10 LD_{50} virulent SFV, or mock-infected with diluent or avirulent SFV (6 × 10^4 p.f.u.).

† Statistical significance was determined using analysis of variance for repeated measures: ‡, $P < 0{\cdot}02$; ‡‡, $P < 0{\cdot}01$; others not significantly different from the mock-infected control. Mean values are shown and figures in parentheses represent the standard error of the mean. Neurotransmitter levels in control mice inoculated only with DI virus were not statistically different from those of mock-infected mice.

[DI SFV encodes no polypeptides itself (Barrett *et al.*, 1984*a*; Barrett & Dimmock, 1985) and is encapsidated in proteins synthesized by infectious virus.] Other groups of mice were mock-infected with diluent, with DI SFV alone or with non-infectious antigen.

One of the features of SFV DI viruses which has emerged only recently is a considerable variability in biological properties both *in vivo* (Barrett & Dimmock, 1984*b*, *d*) and *in vitro* (Barrett *et al.*, 1984*a*; Barrett & Dimmock, 1984*a*, 1986). Accordingly in this work we used two DI virus preparations which, although equally effective in protecting mice against virulent infection lead to different levels of protective immunity (Barrett & Dimmock, 1984*b*). These are referred to as p4 and p13a; the numbers designate the number of undiluted passages (p) they had received in BHK cells (Dimmock & Kennedy, 1978). Infected mice treated with DI p13a were solidly immune to challenge with SFV 3 weeks later and those treated with p4 were as susceptible as previously non-infected animals.

In order to examine neurotransmitter levels mice were killed at intervals after inoculation by gassing with ether, brains were then removed and frozen in a mixture of solid carbon dioxide and methanol. Neurotransmitter assays were performed on whole homogenized brains according to established procedures (Fonnum, 1969; Waddington & Cross, 1978; Cross & Joseph, 1981). Brains were assayed for homovanillic acid (HVA: a metabolite of dopamine), 5-hydroxyindolacetic acid (5HIAA: a metabolite of 5-hydroxytryptamine), glutamate decarboxylase (GAD: which synthesizes γ-aminobutyric acid from glutamate) and choline acetyl transferase (CAT: which catalyses the final step in the production of acetylcholine).

Table 2. *Analysis according to clinical criteria of neurotransmitter changes in mice infected with SFV and treated with DI virus**

	HVA (ng/mg)	5HIAA (ng/mg)	CAT (pmol/mg/h)	GAD (nmol/mg/h)
Mock-infected	1·6	3·0	20·6	50·9
	(0·07)	(0·22)	(1·99)	(1·61)
SFV + DI p4				
+ Clinical signs (6/20)	6·4†	4·5	26·9	39·0
	(1·29)	(0·67)	(4·6)	(8·8)
− Clinical signs (14/20)	2·2	2·4	23·8	36·9
	(0·36)	(0·13)	(1·6)	(8·1)
SFV + DI p13a				
+ Clinical signs (6/20)	8·7†	4·1	21·0	58·4
	(2·16)	(0·47)	(0·76)	(4·08)
− Clinical signs (14/20)	3·7†	2·3	26·3	42·2
	(0·59)	(0·15)	(1·81)	(4·28)

* At 4 days post-infection; data from Table 1.
† $P < 0·002$ compared with mock-infected. Other values were not significantly different.

Mice infected with the ts^+ strain of SFV die on day 5 but when animals are treated with DI SFV most develop an entirely subclinical infection. The latter normally clear infectivity from the brain by 10 days. Brain samples were taken from all groups at day 4 post-infection and from the surviving clinically normal animals at days 12 and 21. Table 1 shows that on day 4 mice infected with virulent SFV alone had levels of HVA elevated by over 300% ($P < 0·002$) (and other data demonstrate that this rise follows the increase in virus multiplication: A. J. Cross *et al.*, unpublished results). A significant increase was also seen in the mean value of all infected mice treated with both DI p4 and p13a. However these data are misleading as the latter groups consist of two different populations: a minority which are not protected and die with disease following its normal course and the majority which are subclinically infected but protected against clinical disease (Barrett & Dimmock, 1984*a*). When DI-treated mice are divided by such clinical criteria a different picture emerges (Table 2): those animals showing signs of disease have very large increases (up to 540%) in HVA, whereas mice protected from clinical disease by DI p4 showed no significant increase. However in infected mice treated with DI p13, HVA increased to over its half-maximum value despite reduction of virus infectivity by 10^2- to 10^6-fold (Barrett *et al.* 1984*b*; data not shown). In these groups none of the other neurotransmitters showed any significant changes irrespective of the status of clinical disease (Table 2). HVA levels returned to normal by 12 days after infection.

Of the other neurotransmitters 5HIAA was elevated on day 4 in mice infected with SFV alone but unchanged in mice treated with DI viruses at any of the times sampled (Tables 1 and 2). CAT was not affected in any of the groups of infected animals. GAD activity was slightly increased in SFV-infected mice ($P < 0·05$) and there was a small (not statistically significant) decrease in mice treated with DI SFV p4. Mice which showed clinical signs and those which did not on day 4 (Table 2) did not differ in GAD levels. However at 12 days there was a very large (up to 250%) decrease in GAD activity although these mice were clinically normal and showed no evidence of infectious virus (data not shown). Surprisingly levels of GAD had returned to normal by day 21. Further observations have not been made, and we do not yet know whether GAD levels are stable or undergo periodic fluctuations.

More detailed analysis (Table 3) of the GAD levels of individual animals at day 12 showed that they had a biphasic distribution and the overall mean in Table 1 obscures just how large was the reduction of GAD activity in the majority of mice. Table 3 shows that 58% (7/12) mice which had been infected with 10 LD_{50} plus DI SFV p4 had titres which averaged at 15% of the values of non-infected mice ($P < 0·001$); likewise 64% (9/14) mice treated with DI virus p13a averaged at 11% ($P < 0·001$). GAD levels in the remaining mice in both groups did not differ from controls. By this stage of infection none of the brains contained virus infectivity and mice

Table 3. *Neurochemical data obtained from individual DI-virus protected mice on day 12 post-infection**

	Mouse no.	GAD (nmol/mg/h)		HVA (ng/mg)		5HIAA (ng/mg)		CAT (pmol/mg/h)	
Mock infected		48·7 (4·77)		1·4 (0·12)		2·4 (0·29)		27·3 (2·91)	
DI SFV p4 + 10 LD_{50}	1	3·2		1·0		0·9		18·9	
	2	3·6		1·0		0·5		13·4	
	3	4·1		2·3		1·4		17·8	
	4	4·1	6·8 (2·28)	1·0	1·4 (0·23)	1·1	1·4 (0·24)	19·1	19·9 (2·2)
	5	5·9		1·0		2·0		14·9	
	6	6·6		1·2		1·6		29·0	
	7	20·2		2·2		2·3		26·2	
	8	42·3		1·5		2·2		26·3	
	9	53·1		0·9		1·8		28·0	
	10	53·1	53·8 (3·4)	NT†	1·3 (0·17)	NT	2·0 (0·24)	27·8	30·0 (1·78)
	11	57·6		1·3		1·5		31·7	
	12	63·0		1·7		2·6		36·2	
DI SFV p13a 10 LD_{50}	1	3·7		1·3		0·7		13·8	
	2	3·9		1·0		1·4		28·5	
	3	3·9		3·4		1·3		34·0	
	4	4·4		1·3		1·6		31·2	
	5	4·8	5·2 (0·49)	0·9	1·4 (0·25)	1·1	1·5 (0·16)	21·3	25·4 (1·94)
	6	4·9		1·3		2·2		24·5	
	7	6·8		0·9		1·4		24·3	
	8	7·2		1·2		1·9		25·4	
	9	7·2		1·7		2·0		25·5	
	10	27·2		1·3		1·1		14·3	
	11	28·6		2·0		2·0		44·3	
	12	38·8	40·7 (6·2)	1·3	1·4 (0·16)	1·8	1·7 (0·12)	37·9	34·0 (5·25)
	13	48·8		1·0		1·8		33·1	
	14	59·9		1·6		1·9		40·5	

* Mice and methods are those described in legend for Table 1.
† NT, Not tested.

were clinically normal (data not shown). By comparison, mice with low GAD activity had normal levels of HVA, 5HIAA and CAT (DI p13a) but the level of CAT in mice treated with DI SFV p4 was reduced ($P < 0{\cdot}02$). Thus, we conclude that the decrease in GAD levels is a specific effect of the DI virus-modulated infection.

It is difficult to design controls for the effect of DI virus in the later time samples as standard virus-treated animals are dead by this time. However a qualitatively different type of infection is seen with an avirulent strain of SFV [derived from A774 (Bradish *et al.*, 1971)] which also invades the CNS but infects oligodendrocytes predominantly and causes a non-lethal demyelination (Suckling *et al.*, 1978; Crouch *et al.*, 1982). Examination of these mice showed similar increases in HVA ($P < 0{\cdot}02$) to those seen with the virulent strain of SFV at 4 days but in contrast to the virulent strain of SFV, 5HIAA concentrations were unchanged throughout the infection (Table 1). By 12 days all neurochemical markers, including GAD, were normal; hence, infection by avirulent SFV differed from the avirulent infection which resulted when infection by the normally lethal strain of SFV was modulated by DI virus.

An increase in HVA concentration (reflecting increased dopamine turnover) has been reported in several animal models of virus encephalitis (Lycke *et al.*, 1970; Ball, 1982) and may be associated with infection of dopaminergic cell bodies (Cross *et al.*, 1984). In contrast, the changes in GAD activity appear more specific to the DI SFV model. The mechanism of such large losses in enzyme activity remains obscure. However, it would seem unlikely that they reflect a lesion of γ-aminobutyric acid-containing neurons, as enzyme activity returns to control values at 21 days after infection. The interest in these data lies in the fact that mice that are protected

from lethal disease by DI virus and appear clinically normal, exhibit severe neurochemical disturbances even after infectious virus has been cleared and in the absence of overt pathology. Such disturbances differ from these seen either in the lethal infection itself or on infection with an avirulent strain of SFV and demonstrate that infection modulated by DI virus is a unique phenomenon as postulated by Huang & Baltimore (1970). It also seems unlikely that host immune responses are responsible for neurochemical changes, as co-infection of mice with standard virus plus DI SFV p13a but not with standard virus plus p4 stimulates protective immunity (Barrett & Dimmock, 1984*b*), yet GAD activity was decreased in mice infected with either DI virus. As these mice show no histopathological abnormalities (Crouch *et al.*, 1982; Barrett *et al.*, 1984*b*) it may be that neurochemical changes parallel alterations in 'luxury functions' seen in mice persistently infected with lymphocytic choriomeningitis virus (Oldstone *et al.*, 1977, 1982, 1984*a*, *b*; Oldstone & Buchmeier, 1982; Southern *et al.*, 1984). Our system differs fundamentally from this as we are rarely able to detect persistent infection (apart perhaps from the neurochemical changes themselves) by assay for infectious virus and never by assay for virus in brain by histochemical or immunochemical staining (Crouch *et al.*, 1982; Barrett *et al.*, 1984*b*; Barrett & Dimmock, 1984*b*; Atkinson *et al.*, 1986). However we have yet to use molecular probes to search for the presence of viral nucleic acid sequences. The changes we observe are transient but measurements were made on whole brain homogenates which could conceal long-term variations in neurotransmitter levels at specific anatomical locations. Our observations also support the hypothesis that viruses could be a contributory factor in some neurological and neuropsychiatric diseases; however a long-term study of mice surviving SFV infection through the intervention of DI virus is essential to investigate any long-term sequelae.

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