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Broad cross-reactivity with marked fine specificity in the cytotoxic T cell response to flaviviruses

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Cytotoxic T (Tc) cells were generated in mice of five H-2 haplotypes against the flaviviruses Kunjin and West Nile (WNV). A panel of recombinant vaccinia viruses which between them expressed cDNA of the entire Kunjin virus genome were used to infect targets. Anti-Kunjin virus responses to determinants derived from non-structural proteins, especially NS3, NS4A and NS4B, were dominant in most mouse strains; usually only one class I major histocompatibility complex (MHC) restriction element was involved. WNV-immune Tc cells showed similar but not identical patterns of antigen recognition to Kunjin virus-immune Tc cells. The extent to which WNV-immune Tc cells recognized Kunjin virus-encoded determinants varied considerably between mice of different MHC haplotypes.

Introduction

Flaviviruses are a family of small, enveloped RNA viruses, responsible for important human diseases such as dengue fever, yellow fever and Japanese encephalitis. They share similar epidemiology, involving replication in both insect and mammalian hosts, and also a similar replication strategy. All flaviviruses have a single strand of positive-sense RNA of approximately 11 kb as their genome, which encodes, through a single open reading frame, 10 viral proteins (Westaway *et al.*, 1985; Rice *et al.*, 1985; Coia *et al.*, 1988). There are three structural proteins, i.e. the core (C) protein and two envelope proteins, envelope (E) and membrane (M), the latter of which is cleaved from the larger matrix precursor molecule (PrM). The genome encodes three large non-structural proteins, NS1, a glycoprotein perhaps involved in viral maturation, and cytosolic proteins NS3, with known protease activity, and NS5, a putative viral polymerase (Chambers *et al.*, 1990). The genome also encodes four smaller polypeptides, NS2A, NS2B, NS4A and NS4B. These have unknown function, and are strongly hydrophobic.

The increased availability of cDNA from various flaviviruses is now enabling detailed study of immune responses to these viruses and identification of determinants for both T and B cells. The study of T cell immunity to flaviviruses has proved difficult, in part because of the poor adaptation of these viruses to laboratory animals and cell lines. However, several groups have reported T cell responses in mice and humans of both helper [major histocompatibility complex (MHC) class II-restricted] and cytotoxic (MHC class I-restricted) types (Monath, 1988; Uren *et al.*, 1987). Recently, Bukowski *et al.* (1989) have reported a $CD8^+$ cytotoxic T (Tc) cell response in a dengue 4 virus-immune human donor which lysed dengue 4 virus- and dengue 2 virus-infected cells, and in cells pulsed with antigen from all four dengue subtypes. Recombinant vaccinia viruses (VVs) mapped at least two determinants, one in E and one in NS2b/NS3/NS4A (Bukowski *et al.*, 1989). This group has also described cytotoxic $CD4^+$ dengue virus cross-reactive clones (Kurane *et al.*, 1989), and mapped the epitopes recognized to NS3 (Kurane *et al.*, 1991). We have previously described a murine Tc cell response to the flavivirus West Nile (WNV) (Müllbacher *et al.*, 1986; Kesson *et al.*, 1987, 1988). WNV belongs to the serological group of flaviviruses that includes Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV), St Louis encephalitis virus and Kunjin virus, all of which

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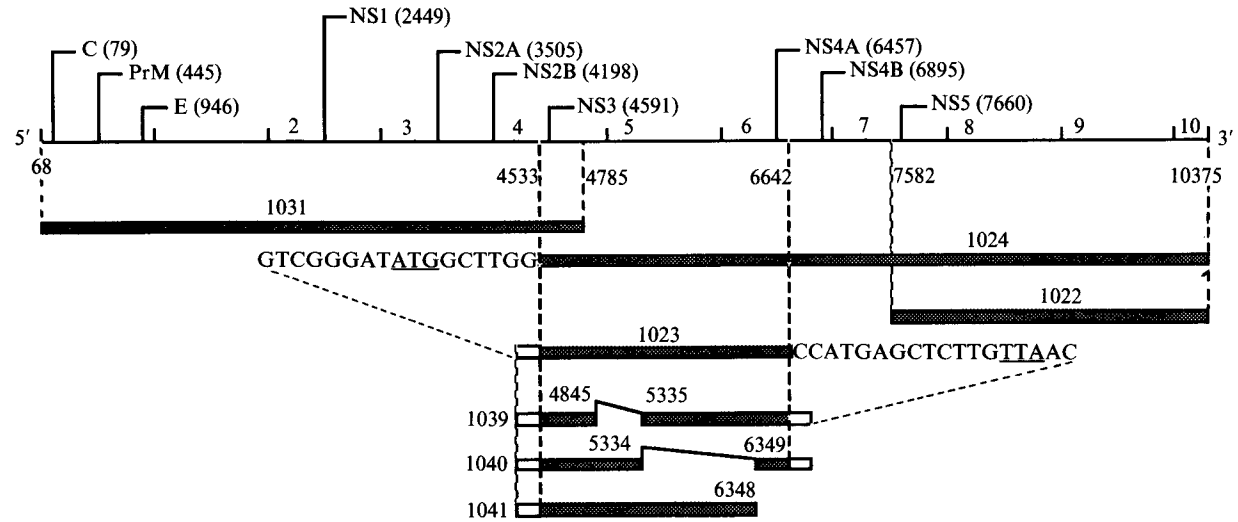


Fig. 1. Kunjin virus–VV recombinants. Kunjin virus genome showing the initial nucleotide position for each gene from the published sequence (Coia *et al.*, 1988).

can cause encephalitis in man. Within this group, WNV and Kunjin virus are particularly closely related, with 93% amino acid identity (Coia *et al.*, 1988).

We have previously described the construction of Kunjin virus–VV (Kunjin–VV) recombinants which between them express cDNA corresponding to the entire Kunjin virus genome (Fig. 1), and demonstrated their use to identify immunogenic determinants for Kunjin virus-immune Tc cells from CBA/H mice (Parrish *et al.*, 1991). We have now completed a comprehensive survey of immunodominant Tc cell determinants in Kunjin virus for mice of five H-2 haplotypes, and identified their restriction elements. We have also studied recognition of these determinants by Tc cells raised against the closely related flavivirus, WNV.

Methods

Mice. Specific pathogen-free mice were supplied by the Animal Breeding Establishment at the John Curtin School of Medical Research, Canberra, Australia. The mice were immunized with virus at age 6 weeks or older.

Flaviviruses. The flaviviruses WNV, Sarafend strain, Kunjin virus, strain MRM 16, MVEV and JEV were provided by Dr I. D. Marshall, and passaged in suckling mouse brains. Supernatant was obtained by centrifugation of a sonicated preparation of mouse brain diluted 1:10 in gelatin-buffered saline, and stored in aliquots at −70 °C for routine use. Virus activity was assayed by titration of p.f.u. on Vero cells, as previously described (Taylor & Marshall, 1975). The titres of virus stocks used were: WNV, 10^9 to 10^{10} p.f.u./ml; Kunjin virus, 1×10^8 to 2×10^8 p.f.u./ml; MVEV, 10^7 p.f.u./ml; JEV, $2{\cdot}5 \times 10^7$ p.f.u./ml.

Kunjin–VV recombinants. The construction of the recombinant VVs used in these experiments has been described in detail (Parrish *et al.*, 1991). Briefly, cDNA fragments corresponding to the Kunjin virus genome were cloned into the VV shuttle vector pBCBO6* downstream of the early/late VV promoter p7·5, and inserted by recombination into the thymidine kinase (TK) gene of the WR strain of VV. Deletion mutations of the NS3/NS4A region of the genome were created as previously described and used for finer delineation of T cell determinants. A TK^- strain of WR (VV-TK^-) was a gift from Dr D. Boyle and was used as the control in Tc cell experiments. The panel of recombinant viruses and the corresponding regions of the Kunjin virus genome are shown in Fig. 1. Virus stocks were prepared by growth in CV1 cells for 48 h after which the cells were pelleted, lysed and sonicated; this crude preparation was used to infect target cells. Stocks were titrated on 143B cells; titres varied from 10^8 to 10^{10} p.f.u. per ml.

Generation of Tc cells. Secondary Kunjin virus- and WNV-immune Tc cells were generated as described (Kesson *et al.*, 1988). Adult mice were injected intravenously (i.v.) with 10^6 p.f.u. of Kunjin virus or WNV. Six to 10 days later (occasionally up to 4 months later) the animals were killed and a single-cell suspension of splenocytes was prepared. One-tenth of the spleen cells were incubated with 2×10^7 p.f.u. Kunjin virus or 5×10^7 p.f.u. WNV in 0·5 ml medium for 1 h, then added back to the rest of the splenocytes. The doses of virus used had been previously determined to boost homologously primed Tc cell populations adequately. After 5 days of culture at 37 °C these cells were used as secondary immune effectors in cytotoxicity assays. The effector cells were demonstrated to be $CD8^+$ by monoclonal antibody and complement lysis for CBA/H mice.

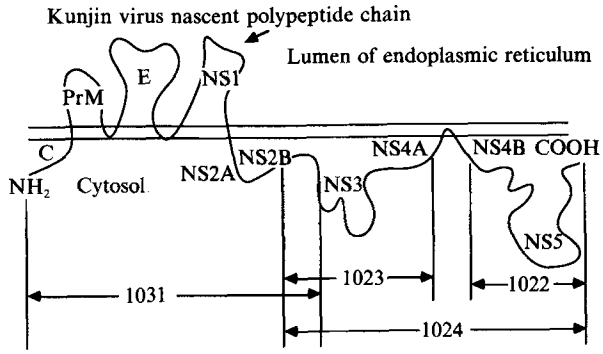
To generate MVEV- and JEV-immune Tc cells, mice were immunized with 10^5 p.f.u. i.v. and 5×10^6 p.f.u. intraperitoneally (i.p.) of MVEV, or 2×10^6 p.f.u. i.v. and 10^7 p.f.u. i.p. of JEV. The spleens were removed as above, and one-tenth of the cells were infected with 5×10^6 p.f.u. MVEV or 10^7 p.f.u. JEV respectively before culture. The virus doses used were the maximum obtainable with available virus stocks.

Target cells for cytotoxicity assays. Peritoneal exudate cells obtained 5 days after i.p. injection of mice with 1 ml of thioglycollate fluid broth (Difco) prepared at 59·6 g/l were used as targets in cytotoxicity assays. These cells were more than 95% activated macrophages as determined

by morphology. Macrophage targets at 4×10^6 cells/ml were incubated with 20 to 50 p.f.u./cell VV simultaneously with ^{51}Cr (100 μCi) for 1 h, and washed twice before use in the assay. For flavivirus-infected targets, macrophages were incubated with 100 to 300 p.f.u./cell WNV, or 25 to 100 p.f.u./cell Kunjin virus in 1 ml medium for 1 h, then incubated in 50 ml spinner culture overnight, before labelling with ^{51}Cr as above.

Cytotoxicity assays. A standard ^{51}Cr release assay was used. Effector cells generated as described above were diluted to the required concentration, usually 10^7 cells per ml, and titrated in a round-bottomed 96-well plate to give triplicates of four threefold dilutions. Target cells (2×10^4) were added per well. Medium without effector cells was added to target cells to give spontaneous ^{51}Cr release control values, and 1% Triton X-100 was added to determine maximum releasable ^{51}Cr. The assay was run at 37 °C in 5% CO_2 for 4 to 6 h, the plates were centrifuged, 100 μl supernatant was harvested from each well and γ radiation was counted. Specific lysis [SL (%)] was calculated as (c.p.m. experimental − c.p.m. spontaneous ^{51}Cr release)/(c.p.m. maximum − c.p.m. spontaneous ^{51}Cr release).

Preparation of EL4 supernatant. Supernatant from the tumour cell line EL4 (a gift from Dr C. Sanderson) was collected after 10^6 EL4 cells per ml had been incubated with 10 ng/ml phorbol myristate acetate for 24 h. The supernatant was filtered, stored at −20 °C and used in the assays at a concentration that gave maximal interleukin-2 (IL2) effect as assayed by IL2-dependent concanavalin A blast proliferation (Warren & Pembery, 1981).



Mouse strain	H-2	Tc cell raised against	1031	1023	1024	1022
CBA/H	$H\text{-}2^k$	Kunjin	±	$+(K^k)$	$+(K^k)$	−
		WNV	+	$+(K^k)$	$+(K^k)$	−
BALB/c	$H\text{-}2^d$	Kunjin	+	$+(K^d)$	$+(K^d)$	−
		WNV	+	−	$+(D/L^d)$	$+(D/L^d)$
C57B16/J	$H\text{-}2^b$	Kunjin	+	−	$+(D^b)$	−
		WNV	$+(D^b)$	−	$+(D^b)$	−
B10.G	$H\text{-}2^q$	Kunjin	−	$+(D/L^q)$	$+(D/L^q)$	−
		WNV	−	$+(D/L^q)$	$+(D/L^q)$	−
SJL/J	$H\text{-}2^s$	Kunjin	+	+	+	+
		WNV	+	+	+	+

Fig. 2. Diagram of proposed Kunjin virus replication strategy, after Coia *et al.* (1988). Summary of Kunjin virus determinants recognized by Kunjin virus- and WNV-immune Tc cells from five mouse strains.

Results

Identification of immunogenic Tc cell determinants in Kunjin virus for mice of five H-2 haplotypes

Secondary Kunjin virus-immune Tc cells were tested against Kunjin–VV recombinant-infected macrophages from mice of various H-2 types. The results of a series of assays are given in Table 1, and summarized in Fig. 2. The recombinant vKV-1031, which expresses both membrane-associated structural proteins as well as the cytosolic protein C and the non-structural cytosolic polypeptides NS2a/2b (Parrish *et al.*, 1991), was recognized weakly by C57BL6/J ($H\text{-}2^b$) Tc cells in association with D^b, by BALB/c ($H\text{-}2^d$) (probably K^d-restricted) and by SJL/J ($H\text{-}2^s$) Tc cells (restriction element not identified). B10.G Tc cells did not recognize Kunjin virus determinants in this construct, and the response in CBA/H mice was of dubious significance. However, for each mouse strain examined, the strongest response was seen with determinants in the non-structural proteins, expressed in the recombinant vKV-1024. This could be further mapped to NS3 (or part of NS2B or NS4A), expressed in vKV-1023, for BALB/c (K^d-restricted), CBA/H (K^k restricted), B10.G (D^q or L^q restricted) and SJL/J. Recombinant vKV-1022 was recognized only by SJL/J Tc cells. C57BL6/J Tc cells recognized vKV-1024 (D^b-restricted), but not vKV-1023 or vKV-1022. This maps the D^b-restricted response to the C-terminal half of NS4A or the N-terminal half of NS4B. These results are summarized in Fig. 2.

Further identification of determinants expressed in vKV-1023

As described previously (Parrish *et al.*, 1991), deletion mutants were made from the cDNA expressed in vKV-1023 and were used to create three new recombinants, vKV-1039, vKV-1040 and vKV-1041. The areas of the Kunjin virus genome expressed by these constructs are shown in Fig. 1. Table 2 shows the results of four experiments using Kunjin virus-immune Tc cells. CBA/H, BALB/c and B10.G Tc cells all showed the same patterns of lysis, lysing targets infected with vKV-1039 and vKV-1040, but not vKV-1041. As seen in Fig. 1, cDNA expressed in vKV-1039 and vKV-1040 but not vKV-1041 corresponds to a 294 nucleotide segment overlapping the cleavage site between NS3 and NS4A; presumably the antigenic determinant(s) are encoded in this region. SJL/J mice show a different pattern, recognizing all three deletion constructs. A 312 nucleotide region at the 5′ end of vKV-1023 is common to all three constructs and presumably encodes the determi-

Table 1. *Lysis by secondary Kunjin virus-immune Tc cells of H-2 matched and mismatched targets infected with Kunjin–VV recombinants*

Effector	Target	H-2 type	SL (%) of macrophage targets infected with*†				
			VV-TK⁻	1031	1024	1023	1022
BALB/c	BALB/c	($K^dD^dL^d$)	4,1	12,7	**31,14‡**	**31,14**	9,4
E:T§	C3H.OH	(K^dD^k)	3,1	11,4	**44,29**	**42,28**	14,7
90:1, 30:1	B10.A	($K^kD^dL^d$)	2, −1	7,2	2, −1	5,3	3,2
CBA/H	CBA/H	(K^kD^k)	16,9	11,5	**46,40**	**46,34**	14,9
E:T	C3H.OH	(K^dD^k)	20,13	17,10	20,13	19,10	21,14
60:1, 20:1	B10.A	($K^kD^dL^d$)	16,6	15,4	**51,42**	**60,54**	16,11
B10.G	B10.G	($K^qD^qL^q$)	12,5	14,8	**60,32**	**62,37**	16,7
E:T	B10.AQR	($K^qD^dL^d$)	5,3	3, −3	3,3	5,0	6,5
40:1, 14:1							
C57B16/J	C57B16/J	(K^bD^b)	8,5	16,7	**29,16**	9,2	12,5
E:T	B10.A(2R)	(K^kD^b)	8,4	11,4	**29,15**	7,4	11,4
50:1, 17:1	B10.A(5R)	($K^bD^dL^d$)	3,0	5,1	5,1	5,3	2, −1
SJL/J	SJL/J	(K^sD^s)	1,2	**12,8**	**31,19**	**22,12**	**22,14**
E:T							
50:1, 17:1							

* Spontaneous ^{51}Cr release was less than 25% except for targets infected with vKV-1031 and B10.A targets where spontaneous release was less than 35%. All targets were also subjected to lysis by VV-immune T cells. All recombinant-infected targets and VV-TK⁻-infected controls were lysed in a VV-specific manner and to an equivalent extent confirming adequate infection of the target by the recombinant VV.

† All results are means of triplicates, with S.E.M. less than 4%.

‡ Lysis which significantly exceeded that of VV-TK⁻-infected control targets is shown in bold type.

§ E:T, Effector:target ratio.

Table 2. *Mapping of antigenic determinant(s) recognised in vKV-1023 by Kunjin virus-immune Tc cells**

Macrophage target	Lysis by CBA/H ($H\text{-}2^k$) Tc cells E:T 100:1, 30:1	Macrophage target	Lysis by BALB/c ($H\text{-}2^d$) Tc cells E:T 120:1, 40:1	Macrophage target	Lysis by B10.G ($H\text{-}2^q$) Tc cells E:T 40:1, 14:1	Macrophage target	Lysis by SJL/J ($H\text{-}2^s$) Tc cells E:T 50:1, 17:1
CBA/H		BALB/c		B10.G		SJL/J	
VV-TK⁻	7,5	VV-TK⁻	9,9	VV-TK⁻	14,5	VV-TK⁻	12,5
vKV-1039	**31,19**	vKV-1039	**27,13**	vKV-1039	**52,31**	vKV-1039	**24,13**
vKV-1040	**32,19**	vKV-1040	**27,12**	vKV-1040	**41,19**	vKV-1040	**20,11**
vKV-1041	7,3	vKV-1041	7,0	vKV-1041	11,9	vKV-1041	**21,12**

* As for Table 1 except that spontaneous ^{51}Cr release was less than 29%.

nants; alternatively, SJL/J Tc cells may respond to more than one determinant in vKV-1023.

Killing of Kunjin virus–VV infected targets by Tc cells generated against the closely related flavivirus, WNV

The fine specificity of viral recognition between two closely related flaviviruses was next investigated. Kunjin virus and WNV have an overall amino acid identity of 93% (Coia *et al.*, 1988).

We investigated discrimination between WNV and Kunjin virus at the level of target recognition by using the panel of Kunjin virus–VV recombinants to infect targets, and generating WNV-immune effector cells from mice of five different H-2 haplotypes, as described above. The results of a series of typical assays are shown in Table 3(*a*). Probably because the laboratory strain of WNV is better adapted to the mouse than is Kunjin virus, Tc cells raised against WNV often showed more killing than those raised against Kunjin virus. In B10.G and C57BL6/J mice, WNV-immune Tc cells showed almost the same pattern of recognition as Kunjin virus-immune Tc cells, although lysis of vKV-1031-infected targets reached significance in comparison to controls only in WNV-immune Tc cells. The dominant K^k-restricted response to vKV-1023 was the same for both

Table 3. *Cross-reactive lysis of Kunjin virus–VV-infected targets by WNV-immune Tc cells*

Effector	Target	H-2 type	SL (%) of macrophage targets infected with*				
			VV-TK⁻	1031	1024	1023	1022
(*a*) BALB/c	BALB/c	($K^dD^dL^d$)	3,2		**39,14**	3,0	**38,15**
(i) E:T	C3H.OH	(K^dD^k)	2,1		5,0	1,0	3,1
16:1, 6:1	B10.A	($K^kD^dL^d$)	4,2		**56,24**	8,0	**56,21**
(ii) E:T	BALB/c	($K^dD^dL^d$)	12,4	**28,21**	**23,26**	15,9	**42,31**
90:1, 30:1							
CBA/H	CBA/H	(K^kD^k)	4,0		**40,26**	**45,30**	6,4
(i) E:T	C3H.OH	(K^dD^k)	6,3		9,3	11,4	7,3
50:1, 16:1	B10.A	($K^kD^dL^d$)	9,4		**55,43**	**60,46**	11,5
(ii) E:T	CBA/H	(K^kD^k)	15,8	**23,16**	**41,29**	**32,24**	15,9
50:1, 16:1							
B10.G	B10.G	($K^qD^qL^q$)	0,0		**33,23**	**34,21**	4,0
(i) E:T	B10.AQR	($K^qD^dL^d$)	2,0		4,2	−3, −3	5,1
50:1, 16:1							
(ii) E:T	B10.G	($K^qD^qL^q$)	11,6	12,6	**34,26**	**33,21**	21,7
50:1, 16:1							
C57B16/J	C57BL6/J	(K^bD^b)	8,4	**25,14**	**26,20**	8,5	7,3
E:T	B10.A(2R)	(K^kD^b)	8,4	**19,11**	**33,21**	9,9	12,8
50:1, 17:1	B10.A(5R)	($K^bD^dL^d$)	6,3	5,1	3,0	5,2	0, −4
SJL/J	SJL/J	(K^sD^s)	−1, −1	**19,11**	**43,32**	**35,25**	**15,7**
E:T							
50:1, 17:1							
			VV-TK⁻	1023	1039	1040	1041
(*b*) CBA/H	CBA/H	(K^kD^k)	15,8	**32,24**	**47,29**	**36,28**	15,11
E:T							
50:1, 17:1							
B10.G	B10.G	($K^qD^qL^q$)	11,6	**21,14**	**40,28**	**27,16**	11,7
E:T							
50:1, 17:1							

* As for Table 1 except that spontaneous ^{51}Cr release was less than 25% and targets infected with vKV-1031 had spontaneous release of less than 32%.

populations in CBA/H mice, but the response to vKV-1031 was stronger in WNV-immune mice. WNV-immune BALB/c Tc cells, however, recognized vKV-1031 and also vKV-1022 (and hence vKV-1024). They did not respond to vKV-1023. The lysis of vKV-1022 was restricted by D^d/L^d. This is a different pattern to Kunjin virus-immune BALB/c Tc cells, which responded to vKV-1023 in association with K^d, and did not respond to vKV-1022. SJL/J WNV-immune Tc cells responded to vKV-1031, vKV-1022, vKV-1023 and vKV-1024, the same pattern as was seen with Kunjin virus-immune SJL/J Tc cells. However, in the anti-Kunjin virus immune response, the lysis of vKV-1022-infected targets was usually greater than that of vKV-1023-infected targets, whereas with the WNV-immune population the reverse pattern was seen. These results are summarized and compared with the Kunjin virus-immune Tc cell response in Fig. 2.

Table 3(*b*) shows the results of two experiments with the deletion mutants vKV-1039, vKV-1040 and vKV-1041, enabling mapping of the determinants responded to by CBA/H and B10.G WNV-immune Tc cells to the same 98 amino acid region recognized by Kunjin virus-immune Tc cells.

Cross-reactivity between Kunjin virus and WNV in the ability to restimulate memory Tc cells primed with the other virus

The surprising finding that BALB/c ($H\text{-}2^d$) WNV-immune Tc cells lysed target cells expressing Kunjin virus determinants (from vKV-1022), whereas Kunjin virus-immune Tc cells did not, prompted us to investigate further the cross-reactivity between these viruses at the level of initiation and restimulation of an immune response. We investigated the ability of WNV and Kunjin to restimulate Tc cell populations primed *in vivo* with the heterologous or homologous virus. Spleens from three animals immunized with Kunjin virus were pooled; half the population was restimulated with WNV and half with Kunjin virus for 5 days *in vitro*. A population of splenocytes from three WNV-immunized animals was treated identically. Aliquots of these four cultures were tested for their ability to lyse macrophage

Table 4. *Cross-reactivity between WNV and Kunjin virus in the ability to restimulate a memory Tc cell population*

Experiment 1. SJL/J Tc cells

Effectors								
Primed *in vivo* with	Boosted *in vitro* with	SL (%) at E:T 30:1 and 10:1 of SJL/J macrophage targets infected with*						
		Uninfected	Kunjin	WNV	VV-TK$^-$	1031	1023	1022
Kunjin	Kunjin	6, 3	23, 13	20, 12	4, 2	20, 13	29, 11	32, 17
Kunjin	WNV	7, 5	32, 25	38, 28	7, 2	24, 18	34, 16	11, 7
WNV	WNV	1, 0	19, 11	37, 26	1, −1	21, 16	39, 23	12, 7
WNV	Kunjin	1, 1	22, 12	20, 11	0, −1	19, 8	26, 9	6, 3

Experiment 2. BALB/c Tc cells

Effector		SL (%) by 1/30 and 1/90 aliquots of culture of BALB/c macrophage targets infected with			
Primed *in vivo* with	Boosted *in vitro* with	VV-TK$^-$	1031	1023	1022
Kunjin	Kunjin	9, 4	20, 6	28, 10	10, 4
Kunjin	WNV	10, 4	8, 2	5, 2	10, 4
WNV	WNV	10, 4	16, 7	10, 3	37, 21
WNV	Kunjin	7, 2	11, 2	5, 2	7, 2

Experiment 3. CBA/H Tc cells

Effectors		SL (%) by 1/30 and 1/90 aliquots of culture of CBA/H macrophage targets infected with						
Primed *in vivo* with	Boosted *in vitro* with	Uninfected	WNV	TK$^-$	1031	1023	1022	Highest E:T ratio
Kunjin	Kunjin	0, 0	15, 10	2, 1	2, 3	28, 14	6, 2	30:1
Kunjin	WNV	2, 1	18, 11	4, 3	9, 6	10, 4	5, 2	20:1
WNV	WNV	6, 3	42, 39	10, 5	17, 12	29, 17	10, 5	20:1
WNV	Kunjin	0, 0	19, 15	3, 2	7, 3	10, 4	2, 0	20:1
WNV	Kunjin + EL4 SN†	6, 3	23, 17	7, 4	4, 3	8, 5	3, 2	32:1
JEV	JEV	0, −1	12, 9	3, 1	1, 0	26, 13	3, 1	25:1
MVEV	MVEV	−1, −1	12, 9	0, 0	−2, −2	2, 1	0, 0	30:1

* Spontaneous ^{51}Cr release was less than 25% for all targets except BALB/c 1031 (35%). S.E.M. was less than 3%.
† SN, Supernatant.

targets infected with WNV, Kunjin virus or the Kunjin–VV recombinants vKV-1031, vKV-1023 and vKV-1022. Three H-2 haplotypes were studied, H-2^s (SJL/J), H-2^d (BALB/c) and H-2^k (CBA/H). The results of three typical experiments are shown in Table 4.

The experiment with homologously boosted H-2^s Tc cell populations confirmed the results above. Kunjin virus-immune Tc cells lysed all targets except uninfected and VV-TK$^-$-infected targets, but vKV-1022 was lysed marginally more efficiently than vKV-1023, whereas WNV-immune Tc cells lysed vKV-1023-infected more efficiently than vKV-1022-infected targets. All four Tc populations lysed targets infected with native flavivirus (either Kunjin virus or WNV). However, WNV infection of macrophage targets was more efficient than that of Kunjin virus, making quantitative comparison impossible. Stimulation by heterologous virus in each case generated Tc cells that recognized the vKV-1031-, 1023- and 1022-encoded determinants.

With H-2^d Tc cells, as expected, WNV failed to restimulate a Kunjin virus-primed population to recognize vKV-1023. In addition, however, Kunjin virus failed to restimulate a WNV-primed population to lyse vKV-1022-infected targets, despite the fact that vKV-1022 expresses the Kunjin virus, not WNV, polypeptide. In each instance, the primed population was restimulated efficiently by the homologous virus to recognize the expected antigen.

The immunodominant determinant that stimulates both Kunjin virus- and WNV-immune CBA/H (H-2^k) Tc

cells is expressed in the construct vKV-1023 is K^k-restricted, and maps to a 98 amino acid sequence surrounding the cleavage site between NS3 and NS4A, a region in which Kunjin virus and WNV differ by only seven amino acids. Table 4 (experiment 3) shows the results of a cross-stimulation experiment. Both memory Tc cell populations failed to respond to restimulation by the heterologous virus and to recognize the immunodominant determinant in vKV-1023, despite good restimulation by the homologous virus. We wondered whether this defective cross-stimulation was due to inadequate cross-reactivity of MHC class II-restricted determinants, as the development of flavivirus Tc cells *in vitro* is dependent on helper T cells (Kesson *et al.*, 1988). However, EL4 supernatant, as a source of helper factors, did not improve the ability of Kunjin virus to boost WNV-primed Tc cells to lyse vKV-1023-infected targets.

Recognition of WNV- and Kunjin virus–VV-infected targets by JEV- and MVEV-immune CBA/H Tc cells

The last two lines of Table 4 show the result of an experiment designed to assess the ability of JEV- and MVEV-immune CBA/H Tc cells to recognize WNV and Kunjin virus determinants. JEV and MVEV Tc cell effectors were generated as described above. We were unable to infect macrophages with our stocks of MVEV and JEV, so we do not know how strongly these Tc cells would have lysed homologously infected targets. Both JEV- and MVEV-immune Tc cells showed weak lysis of WNV-infected targets. In addition, JEV-immune Tc cells efficiently lysed target cells infected with the Kunjin–VV recombinant vKV-1023.

Discussion

The simplicity of the flavivirus genome has enabled us to express polypeptide fragments covering the entire Kunjin virus polypeptide from a series of VV vectors, and to study the preferred regions of the Tc cell response in a more comprehensive fashion than has been possible in most viral systems. Despite the existence in a flavivirus-infected cell of an entirely foreign protein sequence of 3433 amino acids, we found a remarkable concentration of the Tc cell response in each mouse strain to particular determinants, often involving the use of a single class I MHC restriction element. The same limited determinant responsiveness was seen in a primary *in vivo* CBA/H Kunjin virus-immune Tc cell population (Parrish *et al.*, 1991), and so cannot be attributed to artefacts due to the *in vitro* restimulation used here. Strikingly, the dominant response to Kunjin virus in H-2^k, H-2^d and H-2^q mice in each case localized to a 98 amino acid region of the non-structural proteins NS3/NS4A, and in H-2^b mice to an adjacent region NS4A/4B. Mice of the H-2^s haplotype may also recognize epitopes in this region. Identification of peptide determinants will answer the question of whether the same amino acid sequence is involved; however, the fact that the K^k- and K^q-restricted Kunjin virus-derived determinants are recognized by WNV-immune Tc cells, whereas the K^d-restricted determinant is not, suggests that the peptides involved are not identical. The flavivirus polypeptide is encoded in a single open reading frame, causing all flavivirus proteins to be synthesized in equimolar amounts and at the same time in the viral replicative cycle. Thus the concentration of immunodominant viral peptides to a small region of the polypeptide suggests that it is in some way particularly favoured for interaction with the processing machinery which creates peptides for association with class I MHC.

Tc cell responses often show the ability to recognize other closely related viruses, presumably due to shared determinants derived from conserved internal virus sequences (Zweernink *et al.*, 1977; Müllbacher *et al.*, 1986). Dengue 2 virus-specific Tc cells have been described which recognize all four dengue virus subtypes (Bukowski *et al.*, 1989). Cross-reactivity in the immune response to related flaviviruses is of interest because of the high incidence of sequential infection in endemic areas (Fagbami *et al.*, 1988), and because one of the most dramatic consequences of flavivirus infection, dengue haemorrhagic fever and shock syndrome, is due to cross-reactive immunopathology. Our investigation of cross-reactivity between flaviviruses at the level of target cell recognition has revealed both broad cross-reactivity and marked virus-specificity. Kunjin virus and WNV are closely related, with an overall amino acid identity of 93%. The similarity is evenly distributed throughout the viral proteins, being 87% for the C protein, 93% for PrM, 93% for E, and for the non-structural proteins 91% (NS1), 90% (NS2A), 98% (NS2B), 93% (NS3), 93% (NS4A), 89% (NS4B) and 94% (NS5). MVEV and JEV are less closely related, their similarity to Kunjin virus for the published sequence of the structural proteins, NS1, NS2A and NS2B being between 50 and 80% (Coia *et al.*, 1988).

Predictably, WNV- and Kunjin virus-immune Tc cells showed cross-reactive lysis of target cells infected with the heterologous virus, and to a lesser extent JEV- and MVEV-immune Tc cells also recognized Kunjin virus- or WNV-derived determinants. Thus activated effector Tc cells were able to recognize some determinants derived from both closely and more distantly related viruses. However, some differences were seen, such as the preference of Kunjin virus-immune H-2^s Tc cells for a

vKV-1022-encoded determinant, whereas WNV-immune Tc cells preferred vKV-1023. More striking was the response to a Kunjin virus-encoded determinant expressed in vKV-1022 by WNV-immune H-2^d Tc cells, whereas Kunjin virus-immune H-2^d Tc cells did not respond to this determinant. This suggests either that the amino acid sequence of determinants recognized often includes residues at which Kunjin virus and WNV differ, or that there may be differences in the processing of the Kunjin virus and WNV polypeptides, leading to quantitative differences in the amount of each determinant present. Identification of peptide determinants will be important to distinguish between these possibilities.

The fine differences in recognition of related viruses were accentuated in the experiments where memory Tc cells primed with either Kunjin virus or WNV were restimulated with the heterologous virus. These yielded surprising results, such as the example of WNV-immune H-2^d Tc cells which recognized the Kunjin virus sequence expressed in vKV-1022 but were unable to be restimulated by native Kunjin virus to recognize this determinant. In fact, in most cases heterologous virus was poorer in restimulating memory Tc cells than was the virus against which they were primed. At least for the H-2^k system, this defect was not overcome by addition of helper factors. We propose another explanation. Previous experiments have suggested that the antigen concentration threshold required for activation of a Tc cell precursor is higher than that required to enable a Tc effector to lyse a target (Müllbacher & Blanden, 1979). Thus partially cross-reactive determinants may be as good as the priming virus in sensitizing targets for lysis, but poor in their ability to restimulate a memory Tc cell population.

Several authors have suggested that cross-reactive Tc cells may play a role in dengue haemorrhagic fever and dengue shock syndrome (Pang *et al.*, 1982; Bukowski *et al.*, 1989). The variation in the extent of shared determinants dependent on the MHC restricting element involved suggests that some HLA class I alleles may predispose to these conditions. The possibility of immunopathology due to cross-reactive immunity in other flavivirus-associated diseases is raised by the frequent co-circulation of several flaviviruses in endemic areas, where many individuals show serological evidence of multiple flavivirus infections (Fagbami *et al.*, 1988). Tc cells can cause fatal encephalitis in the lymphocytic choriomeningitis (LCM) model in mice (Cole *et al.*, 1972; Oehen *et al.*, 1991), and there is evidence that T cell immunopathology may also be involved in flavivirus encephalitis (Bhatt & Jacoby, 1976; Hotta *et al.*, 1981). On the other hand, T cells are essential for recovery from flavivirus infection (Bhatt & Jacoby, 1976; Hotta *et al.*, 1981; Mathur *et al.*, 1983). A favourable outcome presumably depends on an appropriate balance between the various arms of the immune response and the extent of viral infection; previous exposure to related flaviviruses may affect this balance.

Although immunopathological or protective roles for Tc cells in flavivirus disease remain speculative, the question is of more than academic interest. There is currently considerable interest in the development of flavivirus vaccines, including subunit vaccines. It will be important to design vaccines that do not lead to immunopathological disease enhancement in either homologous or heterologous infection. Immunopathological consequences of a vaccine-induced Tc cell response have been demonstrated in LCM (Oehen *et al.*, 1991). Better understanding of the extent to which antibody, helper T cell and Tc cell responses may be protective or harmful in flavivirus infection is needed. The experiments reported in this paper suggest that non-structural protein determinants dominate the Tc cell response, and emphasize that there may be wide MHC-dependent variation in the extent to which immunity to one flavivirus will augment Tc cell reactivity against another flavivirus.

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