Virology 275, 67–76 (2000)
doi:10.1006/viro.2000.0474, available online at http://www.idealibrary.com on IDEAL®

Characterization of Epitopes for Virus-Neutralizing Monoclonal Antibodies to Ross River Virus E2 Using Phage-Displayed Random Peptide Libraries

J. M. Davies,*[,1] Y.-P. Cai,* R. C. Weir,† and M. J. Rowley*

*Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3168, Australia; and †Division of Biochemistry and Molecular Biology, Faculty of Science, The Australian National University, Canberra, ACT 0200, Australia

Received January 3, 2000; returned to author for revision March 28, 2000; accepted June 1, 2000

Ross River virus (RRV) is the predominant cause of epidemic polyarthritis in Australia, yet the antigenic determinants are not well defined. We aimed to characterize epitope(s) on RRV-E2 for a panel of monoclonal antibodies (MAbs) that recognize overlapping conformational epitopes on the E2 envelope protein of RRV and that neutralize virus infection of cells *in vitro*. Phage-displayed random peptide libraries were probed with the MAbs T1E7, NB3C4, and T10C9 using solution-phase and solid-phase biopanning methods. The peptides VSIFPPA and KTAISPT were selected 15 and 6 times, respectively, by all three of the MAbs using solution-phase biopanning. The peptide LRLPPAP was selected 8 times by NB3C4 using solid-phase biopanning; this peptide shares a trio of amino acids with the peptide VSIFPPA. Phage that expressed the peptides VSIFPPA and LRLPPAP were reactive with T1E7 and/or NB3C4, and phage that expressed the peptides VSIFPPA, LRLPPAP, and KTAISPT partially inhibited the reactivity of T1E7 with RRV. The selected peptides resemble regions of RRV-E2 adjacent to sites mutated in neutralization escape variants of RRV derived by culture in the presence of these MAbs (E2 210–219 and 238–245) and an additional region of E2 172–182. Together these sites represent a conformational epitope of E2 that is informative of cellular contact sites on RRV.

INTRODUCTION

Ross River virus (RRV), a mosquito-borne alphavirus, is the predominant cause of epidemic polyarthritis in Australia and it poses serious medical and economic threats to populations where the virus is endemic. The alphaviruses are enveloped and the viral surfaces are covered in spikes composed of triplets of heterodimers of the envelope proteins E1 and E2 (Smith *et al.,* 1995; Cheng *et al.,* 1995). The Sindbis alphavirus (SIN) has a single N-linked glycosylation site on E2, whereas RRV and other alphaviruses, including the closely related Semliki Forest virus (SFV), possess a second N-linked glycosylation site in E2 (Strauss and Strauss, 1994). The antigenic determinants of RRV and cellular contact sites are yet to be fully defined but a panel of monoclonal antibodies (MAbs) has been generated that recognize conformational epitopes on the outer face of E2 and that neutralize RRV infection *in vitro* (Vrati *et al.,* 1988, 1996). Three of the RRV-specific MAbs, NB3C4, T1E7, and T10C9, recognize overlapping epitopes on E2 that cluster within the region delineated by the two glycosylation sites N200 and N262 (Vrati *et al.,* 1988). These MAbs compete with each other for binding to RRV but their specificity could be distinguished by the location of mutations that occurred in neutralization escape variants derived as a result of culture of RRV in the presence of each of the MAbs. These sites were designated as epitopes a, b1, and b2 (Table 1) (Vrati *et al.,* 1988). However, linear synthetic peptides spanning these sites were incapable of forming the complete epitope for these MAbs (Kerr *et al.,* 1992). Notably, similar regions of E2 of SIN, equine encephalitis virus, and SFV are also antigenic and corresponding synthetic peptides have been used to vaccinate against viral challenge (Strauss *et al.,* 1991; Fernandez *et al.,* 1993; Roehrig, 1993). Thus the panel of MAbs to RRV-E2 define immunodominant epitopes that are conserved in position across related alphaviruses and are of biological importance to the virus.

Phage-displayed random peptide libraries are useful tools for selecting peptides that represent an epitope for a given antibody, since a vast array of different peptides displayed on the surface of bacteriophage can be screened simultaneously (Smith and Petrenko, 1997). Phage-displayed peptides can mimic both linear and discontinuous peptide epitopes as well as epitopes defined by carbohydrate moieties (Hoess *et al.,* 1993) and, as such, this approach is likely to be highly applicable to identification of epitopes for antibodies to the RRV-E2. This study describes the use of phage-display technol-

[1] To whom correspondence and reprint requests should be addressed at current address: The Alfred Hospital, Monash Medical School, Department of Allergy, Asthma, and Clinical Immunology, Commercial Road Prahran, Clayton, Victoria 3181, Australia. Fax: 61-3-9903 0783. E-mail: Janet.Davies@med.monash.edu.au.

0042-6822/00 $35.00

TABLE 1

Mutations in Variants of RRV Grown in the Presence of Anti-RRV-E2 MAbs

Residue of E2	Mutation	MAb	Epitope designation
216	T to I	T10C9	a
234	K to Q, I, N, or E	NB3C4	b1
246	D to V or N	T1E7	b2
248	T to P		
251	R to S		

Note. Data taken from Vrati *et al.,* 1988.

ogy to characterize immunodominant epitopes on the surface of RRV for three virus-neutralizing MAbs: NB3C4, T1E7, and T10C9.

RESULTS

Derivation of the phage clones specifically selected by the MAbs to RRV-E2

After solution-phase biopanning of a phage-displayed random heptapeptide library there were sequenced 43 phage clones isolated for each of NB3C4 and TIE7, 18 for T10C9, and 18 in the absence of selecting antibody. The biopannings were repeated using a solid-phase method and a combination of three random peptide libraries consisting of heptapeptides, cyclic heptapeptides, and dodecapeptides. Following the solid-phase biopanning there were sequenced 23 phage clones for NB3C4, 20 for TIE7, 24 for T10C9, and 29 derived in the absence of selecting antibody. The 171 peptides expressed by the phage clones selected by the MAbs to RRV-E2 were compared using the computer algorithm PILEUP with those of the 47 phage clones derived in the absence of selecting antibody to identify RRV-specific peptides (Fig. 1).

First, we found that, in the absence of selecting antibody, there were 17 phage clones derived by the solution-phase method (with paramagnetic beads) that expressed the motif x_nHKHHx_n and the motif x_nWxxLx_n occurred in five of the peptides derived by the solid-phase method (with polystyrene wells). In addition the peptide C-GQANVNR-C was selected 10 times in the absence of antibody by the solid-phase method. Initial identification and elimination of sets of nuisance peptides that bind components of the biopanning system, such as polystyrene or streptavidin, are recommended (Adey *et al.,* 1995; Capron *et al.,* 1996). We found 66 (39%) of the 171 clones selected by the MAbs to RRV-E2 were similar to the peptide inserts of phage clones derived in the absence of selecting antibodies, which suggests these are specific to components of the biopanning systems and therefore were determined to be nuisance phage clones.

Second, and more important, there were several clusters containing peptides selected exclusively by the MAbs to RRV-E2. A number of peptides (xxKLTxL, VSIFPPA, and KTAISPT) were repeatedly selected by all three MAbs by the solution-phase method. Also, many of the peptides were variants of a predominating sequence (see Fig. 2). Selection of the same phage clones by more than one MAb to RRV is particular to these MAbs and is unlikely to have occurred by cross-contamination, since not all the biopannings were conducted simultaneously. The solid-phase method of biopanning yielded sequences that were not identical to those derived by the solution-phase method and led to more specific selection. Thus LRLPPAP was selected eight times by NB3C4, whereas SSNSELWPLLSA and SAPSSKN were selected three and seven times, respectively, by T1E7. Interestingly, in two adjoining clusters of Fig. 1 the predominant peptides

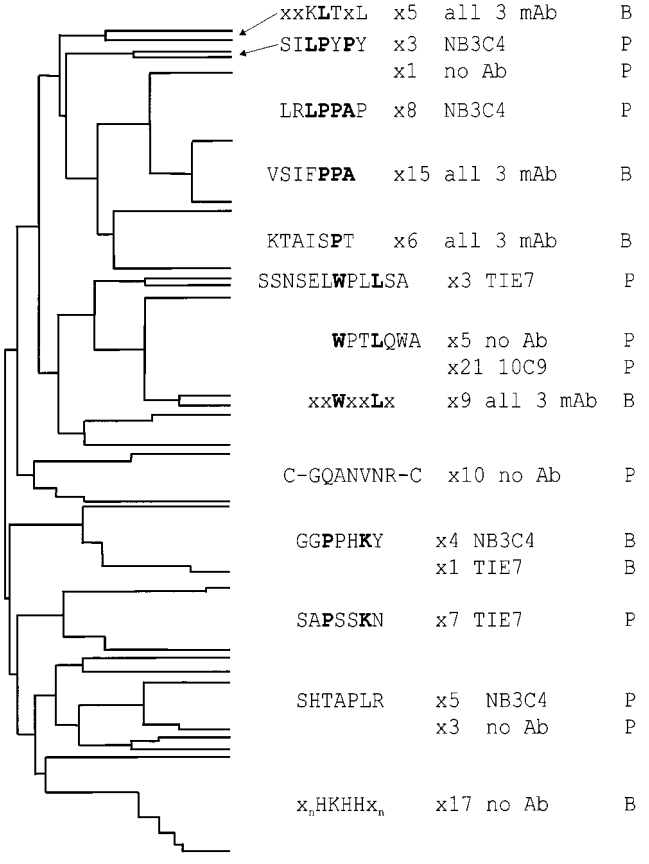

FIG. 1. Phage-displayed peptides selected by MAbs to RRV-E2 align to separate clusters from those selected in the absence of antibody. The sequences of the peptides derived by biopanning in solution-phase with the MAbs bound to anti-mouse IgG-coated paramagnetic beads (B) and in solid-phase with the MAbs bound directly to microtiter plates (P) were compared with those derived by the same methods in the absence of antibody (No Ab) using the multiple sequence alignment algorithm PILEUP. A total of 218 sequences were aligned: 171 from the biopannings with MAbs and 47 from the biopannings without antibody; however, for the sake of clarity, only those peptides or motifs that occurred frequently are shown here. Amino acids that were identical between peptides in adjacent clusters are in bold.

A

NB3C4		T1E7		TI0C9	
.....GGPPHKY.	x3	.....FNFASTS.		...VSIFPPI..	
.....SGPISKY.		.....FGPPSKS.		...VSIFPPA..	
....ESPPPCR..		.....GGPPHKY.		.....LTPFSPK	
....ESWQLWE..		.....IHTPRET.		.....FVPLNHQ	
..IHTS.RF....		.....LQAPNQV.		..MHNLVP...Q	
..FRNELRV....		ADKLSLL.....		KDKLTFV.....	
.AIVT.LPA....		ANRLTLL.....		....HLNGTQP.	
...VTSRGNV...		GNKLTGL.....		....MIPGTQP.	
...VTIFPPR...	x2	..AVSIFPP....		..KTAISPT...	x2
...VSIFPPA...	x2	...VSIFPPA...	x12	....SQSPTLP.	
...VSIFPPI...		..KTAITPT....		.....YHSDAMA	
....SVWPPMG..		..KTAISPT....	x2		
..SLTILRR....		..KTSISPT....	x2		
...KTAISPT...	x2	..ALPGWQP....			
...RTAISPT...		....RYLPLNY..			
...KTSISPT...					
...KTMITPN...					
...LTPL.P.E.L					
....THL.PSQ.L					
...AT...PTQTL					
GNKLSTL.....					
QR.HPAMP.....	x2				
QMSHPAV.....					

B

NB3C4		T1E7		T10C9
.AQPASPD.	x2	.....SAPSSKN.....	x7	HFSQRQEQAMVD
LRLPPAP..	x8	.....LSKDRSS....	*C*	..AQPQSPD...
..LPTNLTQ	*C*	.....PRHQAPT.....	*C*	
.NGST.PKA	*C*	....PETLPYL.....	*C*	
ANTLRSP..		.....GLLSRQP.....		
MLRPHTV..		..TCAVMKPITPW.S.....	x2	
		..NQPNQ.PLVHWES.....		
		SSNSELW.PLL...SA....	x3	
		SDAMGAG		

FIG. 2. Sequences of peptides exclusively derived by each of the MAbs to RRV-E2. (A) The peptides from the solution-phase biopanning in which the MAbs were bound to anti-mouse IgG paramagnetic beads. (B) The peptides from the solid-phase biopanning in which the MAbs were bound directly to microtiter plates. The peptides were aligned by PILEUP. Identical residues between adjacent peptides are in bold. The number of times a particular peptide was derived in a particular biopanning is shown. Peptides marked by *C* were derived from the cysteine-constrained heptapeptide library.

were VSIFPPA and LRLPPAP, which have in common the sequence PPA. Notably, these phage were derived by different biopanning methods. Twenty-one of the phage clones derived using T10C9 by the solid-phase biopanning expressed the peptide WPTLQWA, which was also expressed by five phage clones that were derived in the absence of antibody. By this method only two peptides, AQPQSPD and HFSQRQEQAMVD, were specifically selected for T10C9; these share the motif $QxQx_nD$.

Reactivity of selected phage clones with the MAbs

All of the phage clones selected by the three MAbs by both methods were tested for reactivity with each of the MAbs by direct ELISA and dot-blotting. The phage clones selected in the absence of antibody and eight wild-type phage clones without peptide inserts served as negative controls. Many of the phage clones were also tested by capture ELISA for reactivity with T1E7 and NB3C4. No reactivity was detected for T10C9 with any of the phage clones by any of the assays used, which may reflect low sensitivity of the assays resulting from low antibody affinity, and would be consistent with the small number of specifically selected phage clones isolated using the solid-phase biopanning (see above). None of the phage clones selected in the absence of antibody nor any of those selected with MAbs that were considered to be nuisance peptides were reactive with the MAbs to RRV-E2.

TABLE 2

Reactivity of NB3C4 with the Selected Phage Clones

Phage-displayed peptide[a]	Number of times selected	Selecting MAb[b]	Assay format[c]
..LRLPPAP....	8	N (P)	DB, DE (7/8),[d] CE (2/2)
..VSIFPPA....	15	All 3 (B)	DE, CE
..VSIFPPI....	2	N, T (B)	CE
..VTIFPPR....	2	N (B)	CE
.KTSISPT.....	3	All 3 (B)	CE
.SGPISKY.....	1	N (B)	CE
....SSYMRLW..	1	N (B)	CE
.L.TPLPEL....	1	N (B)	CE
.LPTNLTQ.....C	1	N (P)	DE
SLT.ILRR.....	1	N (B)	CE
SHTAPLR.....	9	N, C, and no Ab (P)	DB (1/5)
......AQPASPD	3	N, C (P)	DB (1/2)
....NNNAPKP..	1	N (B)	CE

[a] Peptides from the constrained library are marked with a *C*.

[b] The MAbs are abbreviated as follows: T, TIE7; N, NB3C4; and C, T10C9. B, the solution-phase biopanning in solution with MAb using anti-mouse Ig beads; P, the solid-phase biopanning with MAb on microtiter plates.

[c] The immunoassays used are abbreviated as follows: DB, dot blot; DE, direct ELISA; and CE, Capture ELISA.

[d] If more than one phage clone expressing the same peptide was tested and the clones gave differing results, then the number of positive clones out of the number tested is given in parentheses.

Reactivity of the selected phage clones with NB3C4

Phage clones that were reactive with NB3C4 are shown in Table 2 and examples of the data obtained by each of the assays are shown in Fig. 3. NB3C4 was strongly reactive by dot-blotting with the eight phage clones that expressed the peptide LRLPPAP and one of five expressing the peptide SHTAPLR (Fig. 3A). NB3C4 was reactive with several phage clones derived by the solid-phase biopanning by direct ELISA; seven of the eight phage expressing the LRLPPAP were among those reactive (Fig. 3B). Notably, the level of reactivity was low by direct ELISA and no reactivity could be detected with phage selected using the solution-phase biopanning, except with a representative phage clone expressing the most frequently selected peptide, VSIFPPA (optical density (OD) = 0.161). In contrast, by capture ELISA, NB3C4 showed strong reactivity with several phage clones derived using the solution-phase method; phage expressing the peptides VSIFPPA, VSIFPPI, VTIFPPR, KTSISPT, and SGPISKY were among those highly reactive (Fig. 3C). Of the phage clones derived in the solid-phase biopanning, each phage tested that expressed the peptide LRLPPAP was highly reactive with NB3C4 by capture ELISA (average OD = 1.4; data not shown).

The peptides that were reactive with NB3C4 frequently contained one or more proline (P) residues, hydroxyl-containing residues (serine, threonine, and to a lesser extent, tyrosine), and long-chain hydrophobic residues. Also many of the reactive peptides contained a basic residue that was mostly arginine. Since phage expressing the peptide LRLPPAP were the most frequently selected by NB3C4 by the solid-phase method and gave the strongest reactivity with NB3C4 by multiple assay formats, this sequence must contain residues important in antigenicity for NB3C4. LRLPPAP and other peptides reactive with NB3C4, LPTNLTQ, LTPLPEL, and SLTILPR are rich in leucine and proline.

Reactivity of the selected phage clones with T1E7

A list of the phage that were reactive with T1E7 is given in Table 3. T1E7 was reactive by dot blot with phage clones derived by the solution-phase biopanning that expressed the peptides FGPPSKS, VSIFPPA, VTSRGNV, SGPISKY, and SLTILRR. T1E7 was also reactive by dot blot with several phage clones derived by the solid-phase method: three of five expressing the peptide SAPSSKN; one expressing the peptide C-PETLPYL-C; and one of three expressing the peptide SSNSELW-PLLSA. Notably, T1E7 reacted with phage selected by NB3C4 using the solution-phase biopanning (i.e., phage expressing the peptides SGPISKY and VTSRGNV). The direct ELISA format was not sensitive enough to give positive reactions between the phage and T1E7, except for a representative phage expressing the peptides VSIF-PPA (OD = 0.266), SSNSELWPLLSA (OD = 0.288), and C-PRHQAPT-C (OD = 0.310). The reactivity detected by capture ELISA was weak with T1E7 and was not significantly above the background binding of phage to microtiter plates without antibody.

Many of the same features that were present in peptides that were reactive with NB3C4 are contained in the phage that reacted with T1E7, that is, proline, residues containing hydroxyl moieties, and hydrophobic residues. However, among the hydroxylated residues there was a preference for serine, which frequently occurred twice or in pairs. Notably, nine of the peptides including SAPSSKN, which was the most frequently selected peptide by the solid-phase method, contained the doublet SK.

Inhibition of reactivity of the MAbs with RRV

Three of the phage clones that were selected at high frequency by the MAbs to RRV-E2 and one phage derived at high frequency in the absence of antibody were tested for their capacity to inhibit the reactivity of T1E7 with RRV. Phage expressing the peptides VSIFPPA, KTAISPT, and LRLPPAP inhibited 63, 42, and 23%, respectively, of the reactivity of T1E7 with RRV, whereas phage without inserts or expressing peptide WPTLQWA derived in the absence of antibody had no effect (Fig. 4). We were unable to show inhibition of NB3C4 reactivity with RRV by

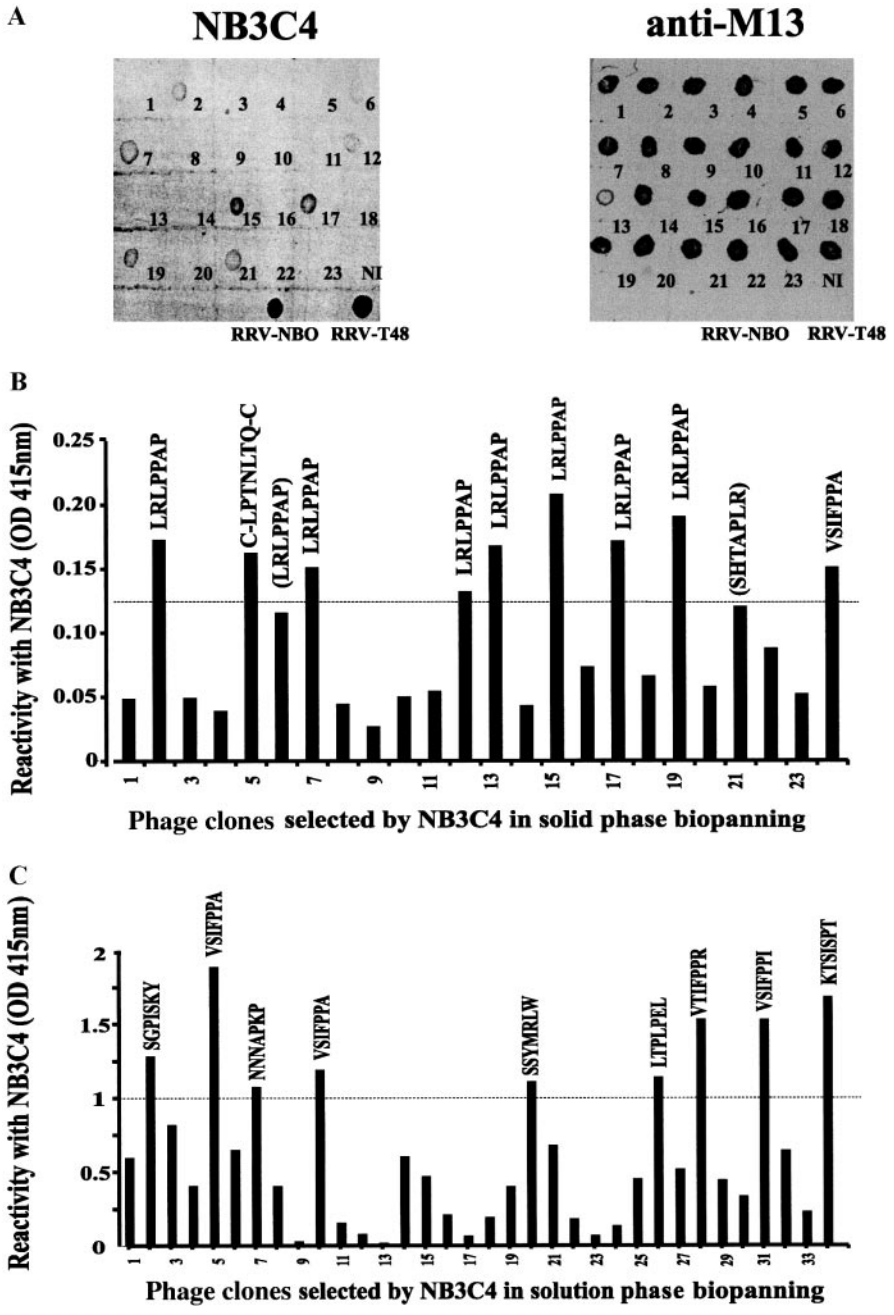

FIG. 3. Reactivity of NB3C4 with representative phage clones by various immunoassays. (A) Reactivity by dot blot of NB3C4 and anti-M13 with RRV and phage selected by NB3C4 in the solid-phase biopanning. NI, a phage clone with no peptide insert. (B) Reactivity by direct ELISA of NB3C4 with phage clones selected by the solid-phase biopanning. The numbers of the phage clones are the same as in (A). The sequences of phage clones that were positive for reactivity, and those that were positive by dot blot, are given. The most frequently selected phage clone from the solution-phase biopanning VSIFPPA was included. (C) Reactivity by capture ELISA of NB3C4 with phage clones derived by the solution-phase biopanning. The sequences of phage clones with positive level of reactivity are shown.

any of the phage clones. This may be the result of technical constraints, since the virus itself did not completely inhibit NB3C4 reactivity with RRV.

Alignment of phage selected by MAbs to RRV

Clusters of similar phage-displayed peptides that were specifically selected by the MAbs to RRV-E2 or reactive with the MAbs to RRV-E2 were aligned to the sequence of RRV-E2 (Fig. 5). The peptides were not identical in primary sequence to any part of RRV-E2 but were similar in amino acid composition to particular regions of RRV-E2 that were between the two N-linked glycosylation sites (N200 and N262) and near the sites of mutations that occurred in variants of RRV derived by culture in the presence of each

TABLE 3

Reactivity of TIE7 with the Selected Phage Clones

Phage-displayed peptide[a]	Number of times selected	Selecting MAb[b]	Assay format[c]
..VSIFP.PA.....	15	All 3 (B)	DB (1/3),[d] DE
..VTSRGNV.....	1	N (B)	DB
.SLT..ILRR.....	1	N (B)	DB
...SGPISKY.....	1	N (B)	DB
...FGPPSKS.....	1	T (B)	DB
...SAPSSKN.....	7	T (P)	DB (3/5)
....SSNSELWPLLSA	3	T (P)	DB (1/3), DE (1/3)
.....PETLPYL..*C*	1	T (P)	DB
...NQPNQDLVHWES.	1	T (P)	DB
PRHQAPT.....*C*	1	T (P)	DE

[a] Peptides from the constrained library are marked with a *C*.

[b] The MAbs are abbreviated as follows: T, T1E7; N, NB3C4; and C, T10C9. B, the solution-phase biopanning in solution with MAb using anti-mouse Ig beads; P, the solid-phase biopanning with MAb on microtiter plates.

[c] The immunoassays used are abbreviated as follows: DB, dot blot; DE, direct ELISA; and CE, Capture ELISA.

[d] If more than one phage clone expressing the same peptide was tested and the clones gave differing results, then the number of positive clones out of the number tested is given in parentheses.

of these MAbs (Table 1). The peptides KTAISPT and VSIFPPA, and variations of these, align with residues 215–219 and 238–245, respectively, of E2. There was an additional region of E2 172–182 outside the region delineated by the two N-linked glycosylation sites with which several of the peptides aligned; these included LRLPPAP, SSNSELSPLLSA, and ANRLTLL. LRL is similar to residues 178–180, whereas PPAP aligns to residues 172–176.

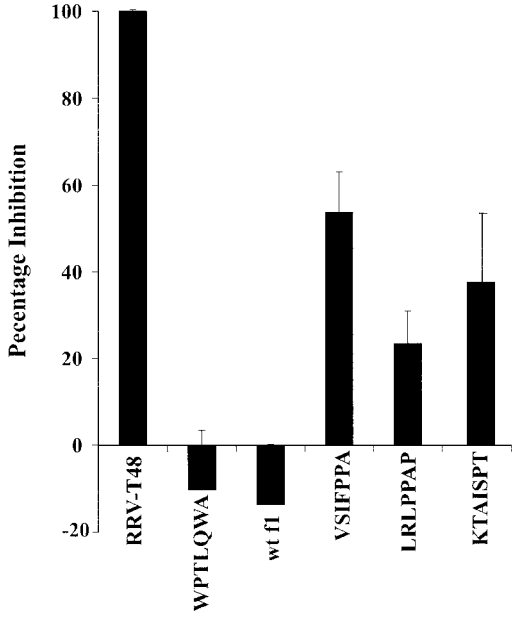

FIG. 4. Partial inhibition of reactivity of T1E7 with RRV by the most frequently selected phage clones. The average percentage inhibition of quadruplicate wells and the SEM are plotted and the peptide sequences displayed by the phage are shown.

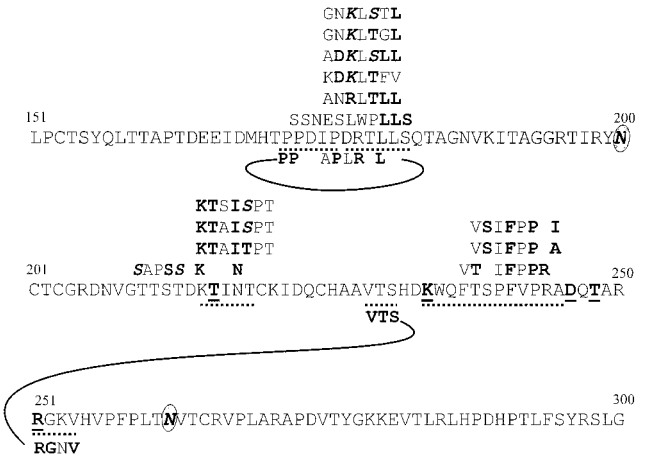

FIG. 5. The sequences of peptides frequently selected and reactive with the MAbs to RRV-E2 match the composition of RRV-E2. Sites of mutations in variants of RRV derived by culture in the presence of the MAbs are in bold and underlined and the two N-linked glycosylation sites are circled and shaded. The regions of E2 with which the peptides align are marked by a dotted underline and the residues of the peptides which match E2 are in bold; similar residues are in bold italics. Parts of two of the peptides matched disparate regions of E2 that might be juxtaposed in the tertiary structure of the virus.

DISCUSSION

We have selected phage clones using a panel of MAbs that neutralize RRV infection *in vitro* from phage-displayed random peptide libraries. By comparison with phage clones derived in the absence of antibody we found that many of the phage-displayed peptides were frequently and exclusively selected by the MAbs to RRV-E2. Many of these were reactive with the MAbs NB3C4 and/or T1E7, and inhibition of the reactivity of T1E7 with RRV was demonstrated with three of the phage clones that were the most frequently selected: VSIFPPA, KTAISPT, and LRLPPAP. Furthermore, the amino acids within the peptides expressed by these frequently selected and reactive phage resemble the amino acid composition of RRV-E2 at sites previously identified as being critical for antibody reactivity. Our data also suggest that there is an additional region outside the glycosylation sites N200 and N262 that participates in binding to these MAbs. Characterization of the antigenic determinants for the MAbs to RRV by phage display is significant because the MAbs are virus-neutralizing. Thus the antigenic sites for these MAbs that can block viral infection of cells could be indicative of cellular binding sites on RRV.

We noted differences in the results for the two biopanning methods. Specifically, the phage-displayed peptides

derived by the solution-phase biopanning were selected by more than one MAb and could react with more than one MAb, whereas the solid-phase biopanning yielded phage-displayed peptides that were specific to each MAb. Selection of the same or similar peptides by the MAbs in the solution-phase biopanning is in accord with the overlapping nature of the epitopes and the cross-inhibitory capacity of these MAbs for reactivity with RRV (Vrati *et al.,* 1988). We also found that reactivity between the phage clones and the selecting MAbs was dependent on the assay format. This is in keeping with previous observations from our laboratory and others and highlights the necessity of testing reactivity of selected phage with the particular antibody by multiple assays (Davies *et al.,* 1999a; Petersen *et al.,* 1995). The level of reactivity between the phage clones and the selecting MAbs to RRV by direct ELISA was low and use of the capture ELISA method increased sensitivity. We demonstrated that the reactivity was specific, since no reactivity was detected with a panel of 46 phage clones, 38 of which displayed other peptides. The issue of low signals in phage ELISA has been addressed by Valadon and Scharff (1996), who developed a capture ELISA in which additional antibody light chains were used to increase the avidity of binding between the MAb and the phage clones.

Notably, we demonstrated inhibition of the reactivity of T1E7 with RRV by the most frequently derived phage clones; however, this inhibition was incomplete, which may be the result of the relatively low molar concentration of the peptide on the phage and difficulties in performing inhibition assays, since the MAbs showed high titer reactivity but very low slopes when titrated against the virus. Alternatively, incomplete inhibition may indicate that the phage-displayed peptides each only partly represents the cognate epitope(s) of E2. Together the amino acids contained within the phage-displayed peptides may each represent parts of the conformational epitope(s) for these MAbs on E2 that would be juxtaposed in the tertiary structure of E2. Similar studies with human serum antibodies to pyruvate dehydrogenase complex (PDC) subunit E2, for which the structure has been solved, have shown that motifs present in selected and reactive phage-displayed peptides were contiguous on the tertiary structure of PDC-E2 and formed a conformational epitope, but arise from discontinuous residues of the protein (Rowley *et al.,* 2000).

The peptides expressed by the phage clones represent regions of RRV-E2 adjacent to point mutations observed in neutralization escape variants derived by culturing RRV in the presence of each of these three MAbs and thus are consistent with previously published data (Vrati *et al.,* 1988). There were three regions with which the peptides aligned with the sequence of E2. Two of these fitted closely with sites of mutations in MAb neutralization escape variants that were designated epitopes a, b1, and b2. The peptide KTAISPT, which was selected frequently and by all three MAbs, aligns to the 216 T to I mutation that was induced by culture of RRV in the presence of T1OC9 and was designated as epitope a. Notably, a natural variant of RRV from a human subject was mutated at residue T 219 to A (Burness *et al.,* 1988), the residue N218 when mutated to R in strains of SIN confers increased neurovirulence, and RRV with N218 mutated to K replicates less efficiently in chick embryo fibroblasts (Kerr *et al.,* 1993). In E2 at 210–213 there is the sequence TTST. Many of the phage-displayed peptides that were reactive with T1E7 contained hydroxylated residues (T, S, or Y), often with a pair of serines, for example SAPSSKN. Thus this region of E2, and hydroxylated residues in particular, may not only form part of the epitope for these MAbs, but may also be of biological significance for the viral infectivity.

A further group of peptides, including VSIFPPA that was isolated 15 times, aligned at amino acids 236–245. This is in accord with sites of mutations in RRV variants cultured in the presence of T1E7 and with a B cell epitope of the most closely related alphavirus SFV, E2 240–255 that includes the peptide PFVPRAD (Fernandez *et al.,* 1993). The third major region of alignment is with amino acids 172–182, and it is notable that this region contains not only PP, which was present in many of the selected peptides, but also the motif LLS that also occurred frequently. The similarity of the peptides VSIFPPA and LRLPPAP, each derived using different biopanning methods, may indicate that these two regions participate in forming the epitope for these MAbs. The first part of the peptide VSIFPPA contains residues (V, S, and F) present in E2 238–245. The residues 172–176 of E2 could be adjacent on the tertiary structure. The LRL part of the peptide LRLPPAP aligns with residues 178–176 RTLL. The R is likely to be important in cellular penetration, since a point mutation in SIN from G to R at 172 resulted in increased neurovirulence (Tucker and Griffin, 1991) and the R is conserved in SFV.

The region of RRV between the N-linked glycosylation sites 200 and 262 was thought to contain not only the epitopes for these virus-neutralizing MAbs but also the cellular receptor binding site on RRV (Vrati *et al.,* 1988; Smith *et al.,* 1995). This region of RRV forms the tip of the spikes of the E1–E2 heterodimers that cover the viral surface (Cheng *et al.,* 1995) and the MAb TI0C9 has been imaged as a complex bound to the tip of the E2 spike in RRV (Smith *et al.,* 1995). Our data suggest that residues outside the two N-linked glycosylation sites are also involved in antibody binding. Interestingly, residues 163–203 are conserved in 51 isolates of RRV taken from mosquitoes and humans infected with RRV (Sammels *et al.,* 1995). If residues critical for contact are conserved, while noncontact residues can vary without deleterious effects on viral propagation, then one might speculate that the residues from 163 onward participate in cellular

contact. This would include the additional region of E2, 172–182, with which several of our phage-displayed peptides aligned. Combining the three major sites at which the immunoreactive phage-displayed peptides selected by the MAbs match RRV-E2, we suggest that PPDIP from 172–182; KTINT from 210–219; and the V, S, and F within 238–245 together form immunodominant antigenic determinants on RRV-E2 and are adjacent in the tertiary conformation of E2.

We conclude that the phage-displayed peptides selected by biopanning phage-displayed random peptide libraries with MAbs to RRV-E2 that neutralize virus infection *in vitro* each partially mimics the binding sites for these MAbs on E2. Moreover, the results of this study provide further information for the identification of the sites on E2 that form the cellular receptor binding site.

MATERIALS AND METHODS

Antibodies and phage libraries

Ascites fluid was obtained from mice injected with the hybridomas T1E7, NB3C4, and T10C9 (Vrati *et al.,* 1988) and the amount of immunoglobulin (Ig) G present was estimated to be 16, 11, and 0.5 mg/ml, respectively. Ascites fluid was also obtained from a negative control mouse injected with sarcoma cells. The PhD-7, PhD-12, and PhD-7C phage display libraries, in which random heptapeptides, dodecapeptides, or cyclic heptapeptides constrained by flanking cysteine residues are inserted at the amino terminal of the minor pIII coat protein of the M13 filamentous phage, were purchased from New England Biolabs (Beverly, MA). Each of the libraries was generated from 10^9 transformants.

Selection of phage clones

Initially, the phage-displayed random heptapeptide library was biopanned with the MAbs T10C9, T1E7, and NB3C4, using a solution-phase method to capture reactive phage clones on anti-mouse IgG-coated paramagnetic beads (Chemicon International, Temecula, CA) previously described (Davies *et al.,* 1999b). In brief, 25 μg of MAb and 10^{11} phage from the random heptapeptide library were incubated with the beads in 2 mg/ml bovine serum albumin (BSA), 0.05% Tween 20, in phosphate-buffered saline (PBS, pH 7.4) overnight. Unbound phage were removed by a series of ten 2-min washes with PBS/BSA/Tween. Captured phage were eluted with 0.1 M glycine–HCl, pH 2.2, and neutralized with 1 M Tris. The phage were negatively selected after the first two rounds by incubation with the beads alone before being amplified and subjected to further rounds of biopanning. For the third round of positive selection 2.5 μg of MAb was used and the captured phage were negatively selected with Ig from a control mouse ascites fluid.

To increase the probability of deriving useful clones with higher affinity, a second method of biopanning was used in which the MAbs were bound directly onto the surface of microtiter plates (solid-phase) in a manner similar to that of Gevorkian *et al.* (1998). When the dodecapeptide and cyclic heptapeptide libraries became available all three phage-displayed libraries were combined and biopanned with each MAb. Sterile microtiter plate wells were coated overnight at 4°C with 1 μg per well of IgG that was purified from the ascites fluids by ammonium sulfate precipitation. The wells were blocked with PBS/BSA/Tween with 0.1% Tween 20. An aliquot of each of the three phage libraries to a total of 2×10^{11} phage particles was added in PBS/BSA/Tween and incubated overnight at 4°C. Unbound phage were washed away and the bound phage were eluted in 0.1 M HCl–glycine, pH 2.3, and immediately neutralized with 1 M Tris–HCl, pH 9. The eluted phage were transferred to wells blocked with BSA and incubated for 2 h at room temperature (RT) as a negative selection step to remove irrelevant phage that bind to the blocked wells. The residual phage were then amplified in *Escherichia coli* ER2537 and purified (Parmley and Smith, 1988). A second and third round of biopanning were conducted. The amount of antibody was reduced 10-fold, the Tween 20 concentration was increased to 0.5%, and the phage were incubated in the MAb-coated wells for only 2 h at RT in the third round of biopanning. Negative selection in the third round was conducted using wells coated with the Ig from the negative control ascites fluid.

After the third round of biopanning, *E. coli* ER2537 were transduced with the eluated phage and plated in semisolid agar. Clones were prepared from individual phage plaques. The ssDNA was extracted using phenol chloroform saturated with TE, ethanol-precipitated, and sequenced with Sequenase Version 2.0 (Amersham, Buckinghamshire, UK) to identify the peptide sequence (Parmley and Smith, 1988).

Biopanning was also conducted by each method in the absence of selecting antibody to deduce irrelevant peptides that bind to components in the biopanning system.

Analysis of peptide sequences

Comparison of phage selected by the MAbs with phage selected in the absence of antibody. The sequences of the peptides expressed by the phage clones selected by the MAbs to RRV-E2 were compared with those from phage derived in the absence of selecting antibody, as described by Davies *et al.* (1999b). The multiple sequence alignment algorithm PILEUP (Feng and Doolittle, 1987) was used to align related peptides into clusters and to generate a guide tree.

Alignment of phage-displayed peptides with RRV-E2. Clusters of similar peptides were aligned in groups with the E2 sequence of RRV-T48 (Dalgarno *et al.,* 1983) using PILEUP as described earlier. The alignments generated

by PILEUP were used as a guide to give the best visible match between the peptides and RRV-E2. The validity of the interpretation of conformational epitopes, based on the location within the cognate antigen of frequently selected amino acids of reactive phage clones, was based on our previous experience in aligning phagotope peptides with antigenic sequences (Konigs *et al.,* 2000; Rowley *et al.,* 2000).

Assessment of reactivity of the selected phage with the MAbs

Dot blot. Two microliters of each phage suspension (10^{12} plaque-forming units (pfu)/ml) was applied to nitrocellulose membranes and allowed to dry. The membranes were blocked for 2 h at RT in diluent containing Tris–HCl (pH 8.0) buffered saline with 1% BSA, or 5% skim milk powder (MP), and 0.5% Tween 20 (TBS/BSA/Tween). The membranes were incubated overnight at 4°C in ascites fluids diluted 1/200 with the addition of 5 μl of wild-type f1 phage (f1; 5×10^{13} pfu/ml) and 20 μl of *E. coli* ER2537 lysate/ml (Cook *et al.,* 1998). The membranes were washed in diluent and incubated with anti-mouse Ig conjugated to horseradish peroxidase (HRP; AMRAD, Melbourne, Australia) at 1/2000 in diluent without Tween 20 for 4 h at RT. A MAb to M13 phage (anti-M13; Pharmacia, Uppsala, Sweden) was used at 1/2000 to confirm the presence of phage on a control square. The squares were washed in diluent without Tween and then finally in PBS before being developed with 1,4-chloronaphthol in 20% methanol in PBS with 0.2% H_2O_2.

Direct ELISA. Microtiter plate wells (Nunc Maxisorp F) were coated in duplicate with 5 μl of phage suspension in 100 μl of PBS. The wells were blocked with 300 μl of diluent that contained 1% milk powder in PBS/Tween and washed three times with TBS/Tween. Tween 20 was used at 0.11. f01 ELISA. The ascites fluids were incubated in diluent with the addition of f1 and *E. coli* lysate, as described earlier, for 2 h at RT or overnight at 4°C. The plates were washed three times with TBS/Tween and three times with water. The wells were incubated with anti-mouse Ig-HRP at 1/2000 or anti-sheep Ig-HRP at 1/5000 for 4 h at RT before being washed six times with water. The ELISA was developed using 0.5 mg/ml 2,2 azino-di-[(3-ethyl-benzthioazoline sulfonate-6)] in 0.03 M citric acid, 0.04 M Na_2HPO_4, and 0.003% H_2O_2, and the OD was read at 415 nm. A cutoff above which reactivity was designated positive was set at 2 standard deviations above the mean of 46 phage clones that either lacked inserts or expressed peptides derived in the absence of antibody; for NB3C4 and T1E7 the cutoffs were 0.103 and 0.266, respectively.

Capture ELISA. The capture ELISA was performed as described by Davies *et al.* (1999b) with the following modifications: 1 μg of purified IgG was used to coat the microtiter plate wells and the sheep antiserum to M13 used to detect captured phage was diluted 1:5000. Alternatively, rabbit anti-fd (Sigma Chemical Company, St. Louis, MO) was used at 1/2000 with anti-rabbit-Ig conjugated to HRP at 1/2000 as secondary antibody. The cutoff for a positive reaction was set at 1.0 OD units at 415 nm, which was above the value obtained for 30 phage clones that were captured on the plates in the absence of antibody.

Inhibition ELISA. The inhibition ELISA was performed as described in Davies *et al.* (1999a). Microtiter plates were coated with purified RRV-T48 diluted 1/2000 in 100 μl per well of sterile PBS and held overnight at 4°C. The plates were blocked as described earlier. T1E7 was diluted 1/250,000 in diluent with 10 μl of ER2537 lysate and 1 μl of f1/ml. Tubes with inhibitor diluted in the antibody solution were prepared, mixed by inversion, and incubated at RT for 6 h. Inhibitors were tested in quadruplicate at 50 μl of phage clone per 500 μl, and 1 μl per 500 μl for RRV T48. The plates were then washed and 100 μl of antibody/inhibitor suspension was transferred to the wells and incubated overnight. The ELISA procedure was then continued as described earlier. Inhibition was determined as the percentage reduction in reactivity relative to a set of adjacent wells lacking inhibitor.

ACKNOWLEDGMENTS

This work was supported by the Australian Rotary Health Research Fund for Ross River fever. We thank Dr. J. R. E. Fraser (Monash University) and Dr. A. Subhrier (Vaccine Technology, Queensland Institute of Medical Research, Brisbane, Australia) for valuable discussions.

REFERENCES

Adey, N. B., Mataragnon, A. H., Rider, J. E., Carter, J. M., and Kay, B. K. (1995). Characterization of phage that bind plastic from phage-displayed random peptide libraries. *Gene* **156**, 27–31.

Burness, A. T. H., Pardoe, S. G., Faragher, S. G., Vrati, S., and Dalgarno, L. (1988). Genetic stability of Ross River virus during epidemic spread in nonimmune humans. *Virology* **167,** 639–643.

Caparon, M. H., Ciechi, P. A. D., Devine, C. S., Olins, P. O., and Lee, S. C. (1995). Analysis of novel streptavidin-binding peptides, identified using a phage display library, shows that amino acids external to perfectly conserved consensus sequence and to the presented peptides contribute to binding. *Mol. Div.* **1,** 241–246.

Cheng, R. H., Kuhn, R. J., Olson, N. H., Rossmann, M. G., Choi, H. K., Smith, T. J., and Baker, T. S. (1995). Nucleocapsid and glycoprotein organization in an enveloped virus. *Cell* **80,** 621–630.

Cook, A. D., Davies, J. M., Myers, M., Mackay, I. R., and Rowley, M. J. (1998). Identification of a mimotope to the type II collagen monoclonal antibody CII-C1 using phage display technology. *J. Autoimmun.* **11,** 205–211.

Dalgarno, L., Rice, C. M., and Strauss, J. H. (1983). Ross River Virus 26 S RNA: Complete nucleotide sequence and deduced sequence of the encoded structural proteins. *Virology* **129,** 170–187.

Davies, J. M., Rowley, M. J., and Mackay, I. R. (1999a). Phagotopes derived by antibody screening of phage-displayed random peptide libraries vary in immunoreactivity: studies with an exemplary monoclonal antibody, CII-C1, to type II collagen. *Immunol. Cell Biol.* **77,** 483–490.

Davies, J. M., Scealy, M., Cai, Y.-P., Whisstock, J., Mackay, I. R., and Rowley, M. J. (1999b). Multiple alignment and sorting of peptides derived from phage-displayed random peptide libraries with polyclonal sera allows discrimination of relevant phagotopes. *Mol. Immunol.* **36,** 659–667.

Feng, D.-F., and Doolittle, R. F. (1987). Progressive sequence alignment as a prerequisite to correct phylogenetic trees. *J. Mol. Evol.* **25,** 351–360.

Fernandez, I. M., Snijders, A., Benaisssa-Trouw, B. J., Harmsen, M., Snippe, H., and Kraaijeveld, C. A. (1993). Influence of epitope polarity and adjuvants on the immunogenicity and efficacy of a synthetic peptide vaccine against Semliki Forest Virus. *J. Virol.* **67,** 5843–5848.

Gevorkian, G., Manoutcharian, K., Almagro, J. C., Govezensky, T., and Dominguez, V. (1998). Identification of autoimmune thrombocytopenic purpura-related epitopes using phage-display peptide library. *Clin. Immunol. Immunopathol.* **86,** 305–309.

Hoess, R., Brinkmann, U., Handel, T., and Pastan, I. (1993). Identification of a peptide which binds to the carbohydrate-specific monoclonal antibody B3. *Gene* **128,** 43–49.

Kerr, P. J., Fitzgerald, S., Tregear, G. W., Dalgarno, L., and Weir, R. C. (1992). Characterization of a major neutralization domain of Ross River virus using anti-viral and anti-peptide antibodies. *Virology* **187,** 338–342.

Kerr, P. J., Weir, R. C., and Dalgarno, L. (1993). Ross River Virus variants selected during passage in chick embryo fibroblasts: Serological, genetic, and biological changes. *Virology* **193,** 446–449.

Konigs, C., Rowley, M. J., Thompson, P., Myers, M. A., Scealy, M., Davies, J. M., Wu, L., Dietrich, U., Mackay, C. R., and Mackay, I. R. (2000). Monoclonal antibody screening of a phage-displayed random peptide library reveals mimotopes of chemokine receptor CCR5: Implication for the tertiary structure of the receptor, and for an N-terminal binding site for HIV-1 gp120. *Eur. J. Immunol.* **30,** 1162–1171.

Parmley, S. F., and Smith, G. P. (1988). Antibody-selectable filamentous fd phage vectors: Affinity purification of target genes. *Gene* **73,** 305–318.

Petersen, G., Song, D., Barbara, H. D., Oldenburg, I., and Bautz, E. K. F. (1995). Mapping of linear epitopes recognized by monoclonal antibodies with gene-fragment phage display libraries. *Mol. Gen. Genet.* **249,** 1–7.

Roehrig, J. T. (1993). Immunogens of encephalitis viruses. *Vet. Microbiol.* **37,** 273–284.

Rowley, M. J., Scealy, M., Whisstock, J., Jois, J., Wijeyewickrema, L., and Mackay, I. (2000). Identification of the major immunodominant epitope of the pyruvate dehydrogenase complex-E2 (PDC-E2) in primary biliary cirrhosis using phage display. *J. Immunol.* **164,** 3413–3419.

Sammels, L. M., Coelen, R. J., Lindsay, M. D., and Mackenzie, J. S. (1995). Geographic distribution and evolution of Ross River Virus in Australia and the Pacific Islands. *Virology* **212,** 20–29.

Smith, G. P., and Pettenko, V. A. (1997). Phage display. *Chem. Rev.* **97,** 391–410.

Smith, T. J., Cheng, H., Olson, N. H., Peterson, P., Chase, E., Kuhn, R. J., and Baker, T. S. (1995). Putative receptor binding sites on alphaviruses as visualized by cryoelectron microscopy. *Proc. Natl. Acad. Sci. USA* **92,** 10648–10652.

Strauss, E. G., Stec, D. S., Schmaljohn, A. L., and Strauss, J. H. (1991). Identification of antigenically important domains in the glycoproteins of Sindbis Virus by analysis of antibody escape variants. *J. Virol.* **65,** 4654–4664.

Strauss, J. S., and Strauss E. G. (1994). The alphaviruses: Gene expression, replication, and evolution. *Microbiol. Rev.* **58,** 491–562.

Tucker, P. C., and Griffin, D. E. (1991). Mechanism of altered Sindbis virus neurovirulence associated with a single-amino-acid change in the E2 glycoprotein. *J. Virol.* **65,** 1551–1557.

Valadon, P., and Scharff, M. D. (1996). Enhancement of ELISAs for screening peptides in epitope phage display libraries. *J. Immunol. Methods* **197,** 171–179.

Vrati, S., Fernon, C. A., Dalgarno, L., and Weir, R. C. (1988). Location of a major antigenic site involved in Ross River virus neutralization. *Virology* **162,** 346–353.

Vrati, S., Kerr, P. J., Weir, R. C., and Dalgarno, L. (1996). Entry kinetics and mouse virulence of Ross River Virus mutants altered in neutralization epitopes. *J. Virol.* **70,** 1745–1750.