JOURNAL OF VIROLOGY, Mar. 1996, p. 1745–1750 Vol. 70, No. 3
0022-538X/96/$04.00+0

Entry Kinetics and Mouse Virulence of Ross River Virus Mutants Altered in Neutralization Epitopes

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Received 11 August 1995/Accepted 17 November 1995

Previously we identified the locations of three neutralization epitopes (a, b1, and b2) of Ross River virus (RRV) by sequencing a number of variants resistant to monoclonal antibody neutralization which were found to have single amino acid substitutions in the E2 protein (S. Vrati, C. A. Fernon, L. Dalgarno, and R. C. Weir, Virology 162:346–353, 1988). We have now studied the biological properties of these variants in BHK cells and their virulence in mice. While variants altered in epitopes a and/or b1 showed no differences, variants altered in epitope b2, including a triple variant altered in epitopes a, b1, and b2, showed rapid penetration but retarded kinetics of growth and RNA and protein synthesis in BHK cells compared with RRV T48, the parent virus. Variants altered in epitopes a and/or b1 showed no change in mouse virulence. However, two of the six epitope b2 variants examined had attenuated mouse virulence. They had a four- to fivefold-higher 50% lethal dose (LD_{50}), although no change in the average survival time of infected mice was observed. These variants grew to titers in mouse tissues similar to those of RRV T48. The LD_{50} of the triple variant was unchanged, but infected mice had an increased average survival time. This variant produced lower levels of viremia in infected mice. On the basis of these findings we propose that both the receptor binding site and neutralization epitopes of RRV are nearby or in the same domain of the E2 protein.

Ross River virus (RRV) is a mosquito-borne alphavirus which is responsible for annual outbreaks of polyarthritis in humans in Australia (7, 27). In nature, RRV exists as a number of distinct antigenic types which differ in mouse virulence (9, 12). For example, RRV T48, the prototype strain, which was isolated at Townsville in northern Queensland, is mouse virulent whereas RRV NB5092, isolated at Nelson Bay in coastal New South Wales, is mouse avirulent (10, 22).

The question of what properties of a virus determine its degree of virulence is important for developing effective measures for control of the virus. Two important biological properties that can affect the virulence of a virus are its growth rate and tissue tropism. These properties can be altered, at least in part, by changes in envelope proteins that are involved in receptor recognition, virus attachment and penetration, and membrane fusion to initiate the infection (for a review, see reference 41). Studies with alphaviruses, such as Sindbis virus (26, 28, 33, 36, 40), Venezuelan equine encephalitis (VEE) virus (16, 19), Semliki Forest virus (13, 35), and RRV (22, 42), have also demonstrated that amino acid changes in envelope proteins can affect virus virulence. However, the mechanism by which these changes affect alphavirus virulence is not understood, although studies with Sindbis virus (1, 5, 25, 33) and VEE virus (16, 19) have shown an association between reduced mouse virulence and rapid penetration of BHK cells.

We have previously identified three neutralization epitopes in the E2 protein of RRV T48 by sequencing a number of mutants that are resistant to monoclonal antibody neutralization (43). These are located on or around amino acids 216 (epitope a), 232 and 234 (epitope b1), and 246, 248, and 251 (epitope b2). Nucleotide sequencing of the structural-protein genes of RRV neutralization-resistant variants demonstrated that single amino acid substitutions had occurred in the E2 proteins of these variants (43). Similarly selected neutralization-resistant variants of reovirus (2) and rabies (6, 29, 37), mumps (20), Sindbis (26, 36), and VEE (15) viruses have been shown to express altered virulence in experimental animals. Genetically characterized neutralization-resistant mutants of RRV with single amino acid substitutions in the E2 protein could, therefore, be useful in elucidating the role of the protein, in particular, the role of the neutralization domain, in early virus-cell interactions and in determining viral virulence. In this paper we report on the biological properties, such as growth and entry kinetics, of the neutralization-resistant variants of RRV T48 in BHK cells. On the basis of our study of the mouse virulence of these variants, we also attempt to correlate the changes in the biological properties of the virus to already-defined mutations in the E2 protein.

MATERIALS AND METHODS

Virus strains. The prototype strain of RRV, T48, (4) was obtained from the Yale Arbovirus Research Unit. It had been passaged 10 times in mice when it was received. The virus was plaque purified on Vero cell monolayers before use.

Neutralization-resistant variants of RRV T48 have been described previously (43). Amino acid substitutions in the variants, compared with RRV T48, and their locations in the E2 protein are shown in Table 1.

Virus titrations were done by plaque formation on Vero cell monolayers (24).

Cell culture. BHK-21 cells were grown in Glasgow-modified Eagle's medium, and Vero cells were grown in medium 199-lactalbumin hydrolysate supplemented with 10% heat-inactivated bovine serum (24).

Penetration assay. The rate of virus penetration into the cells was measured in terms of the rate at which resistance to neutralization by added polyclonal antibody is acquired by infecting virus (1). Briefly, BHK monolayers in 60-mm-diameter dishes were infected with approximately 100 PFU of virus and incubated with intermittent shaking at 36°C. At various times after infection the inoculum was removed by aspiration and 0.5 ml of polyclonal anti-RRV T48 immune ascitic fluid (diluted 1:10 in Hanks' balanced salt solution [HBSS]) was added. After 10 min at 36°C the ascitic fluid was aspirated, and the cell monolayers were washed twice with phosphate-buffered saline (PBS) and overlaid for plaque development. The number of plaques obtained after 60 min of adsorption

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and the PBS wash but no immune ascitic fluid treatment was taken as 100%. The number of plaques obtained in this manner was equal to that found by using the normal plaque assay conditions, in which the inoculum is not removed by washing. Each assay was performed in triplicate.

Viral RNA and protein synthesis. Rates of viral RNA synthesis were estimated by incubation of infected cell monolayers for 2 h with [5′-^{3}H]uridine (10 μCi/ml) in Eagle's modified essential medium containing 5 μg of actinomycin D per ml (24). Monolayers were dissociated with 1% (wt/vol) sodium dodecyl sulfate (SDS), precipitated with 5% trichloroacetic acid on glass fiber discs, and counted for radioactivity. Proteins in infected cells were labeled by incubating the monolayers in Eagle's modified essential medium containing 1/10 of the normal level of amino acids and 100 μCi of ^{3}H-amino acid mixture per ml. Monolayers were washed twice with PBS and dissociated in 200 μl of 1% SDS. Cell extracts were electrophoresed on a 10 to 20% gradient polyacrylamide gel.

Estimation of virulence in mice. The 50% lethal dose (LD_{50}) of the virus and average survival time (AST) of infected mice were used to define the virulence of RRV T48 and the neutralization-resistant variants. Mice of either sex from an outbred line (Walter and Eliza Hall Institute [WEHI]) were used. For LD_{50} determination, 30-μl samples of 10-fold virus dilutions in HBSS were injected intraperitoneally into day-old or week-old mice, with 8 to 10 littermates per dilution. LD_{50}s were calculated according to the method of Reed and Muench (30). The incidence of clinical signs and deaths was recorded every day. Clinical signs included waddling gait (moderate signs) and flaccid paralysis (severe signs). The AST is the arithmetic mean of the life span of all mice that died during the course of a titration regardless of dose, since for most RRV strains the time of death was independent of the dose (39).

Virus growth in mice. WEHI mice of either sex were injected intraperitoneally with approximately 100 PFU of virus. Groups of three mice were sacrificed at each time point by cervical dislocation; tissues were removed and stored at −70°C. Blood was collected from the heart with heparinized capillary tubes and diluted 1:10 in HBSS before being frozen at −70°C. For virus assay, tissues were homogenized in a suspension in ice-cold HBSS with a Teflon-coated glass homogenizer attached to a Virtis homogenizer. The virus titer in each tissue was calculated as the average of titers for individual mice at each time point.

RESULTS

Growth kinetics of RRV T48 antigenic variants in BHK cells. The growth of neutralization-resistant variants was compared with that of RRV T48 to examine whether a change in the neutralization domain affected virus receptor recognition and attachment or penetration in ways that may be reflected in virus growth in cultured cells. Variants altered in epitopes a and b1 did not show any change in growth pattern (data not shown). However, all the four epitope b2 variants that were examined showed retarded growth during the early phase of infection. The titers were reproducibly found to be approximately 10-fold lower at least up to 7 h postinfection, after which the difference was less marked, and at 24 h postinfection the variants and RRV T48 had reached similar titers (Fig. 1). That changes in the b2 epitope alone affected the growth kinetics of virus in BHK cells was further demonstrated by comparing the growth of Tv147 (altered in epitopes a and b1) and Tv161 (altered in epitopes a, b1, and b2) with that of RRV T48 (Fig. 2). While variant Tv147 had insignificant differences in growth kinetics compared with RRV T48, Tv161 grew far more slowly. The latent periods were, however, apparently similar.

Penetration of epitope b2 variants into BHK cells. The initially retarded growth of epitope b2 variants in BHK cells suggested that the amino acid change was more likely to affect the initial events of infection (i.e., virus-cell interactions) than intracellular replication. To determine whether a lesion in the virus-cell interaction of the epitope b2 variants existed, we compared the rates of penetration of BHK cells by RRV T48 and the variants as described in Materials and Methods. While variants altered in epitope a or b1 had penetration rates similar to that of RRV T48 (data not shown), the epitope b2 variants penetrated the cells faster than RRV T48 (Fig. 3). This appears paradoxical in relation to the earlier observation that the epitope b2 variants showed initially retarded growth in BHK cells. It should, however, be noted that the method used for these experiments measures the rate at which resistance to neutralization by polyclonal antibody is acquired by infecting virus, and this may not necessarily reflect the rate of virus penetration.

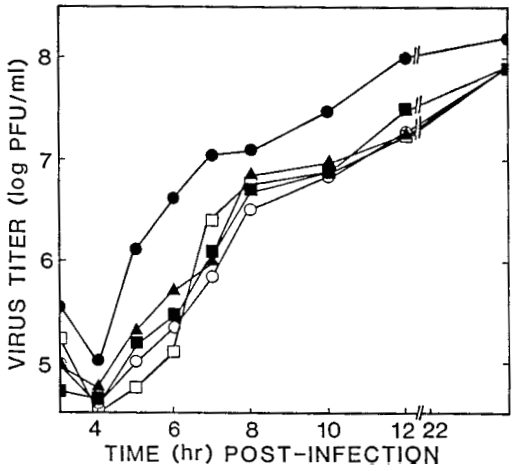

FIG. 1. Growth kinetics of neutralization-resistant variants of RRV T48 in BHK cells. BHK cell monolayers were infected with variant or with RRV T48 at a multiplicity of infection of ~1 and incubated at 36°C. At various intervals samples were removed, diluted in HBSS, and stored at −70°C. The extracellular virus titers were assayed in duplicate. ●, RRV T48; ▲, Tv1; ■, Tv2; ○, Tv3; □, Tv5.

Viral RNA and protein synthesis in BHK cells infected with antigenic variants. The kinetics of actinomycin D-resistant RNA synthesis in BHK cells infected with RRV T48 or Tv147 (altered in epitopes a and b1) were similar. However, cells infected with Tv161 (altered in epitopes a, b1, and b2) showed initially retarded RNA synthesis (Fig. 4). The RNA synthesis rates at later stages of growth showed no difference.

The kinetics of viral protein synthesis was studied by examining virus-specific polypeptide synthesis in infected BHK cells. At 3 to 5 h postinfection, RRV T48-infected BHK cells synthesized only two virus-specific polypeptides, p80 and capsid; none of the epitope b2 variants showed any virus-specific polypeptide synthesis at this time (Fig. 5; data for Tv51 and Tv59 not shown). At 7 to 9 and 23 to 25 h postinfection cells infected with RRV T48 or the variants showed labeling of p110, p95, p80, pE2, E1, E2, and C (Fig. 5); E3 was detected after longer exposure (data not shown). The mobilities of corresponding viral polypeptides were identical in all instances except for Tv1 (Fig. 5), Tv51, and Tv59 (data not shown); in cells infected with these variants, pE2 migrated more slowly

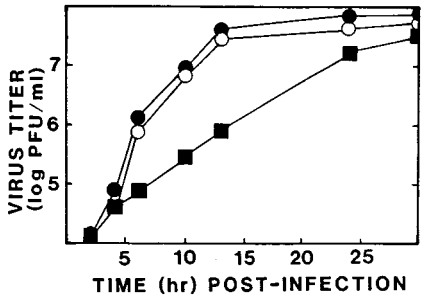

FIG. 2. Growth kinetics of neutralization-resistant variants of RRV T48 in BHK cells. BHK cell monolayers were infected with variants or with RRV T48 at a multiplicity of infection of ~1 and incubated at 36°C. At various intervals samples were removed, diluted in HBSS, and stored at −70°C. The extracellular virus titers were assayed in duplicate. ●, RRV T48; ○, Tv147; ■, Tv161.

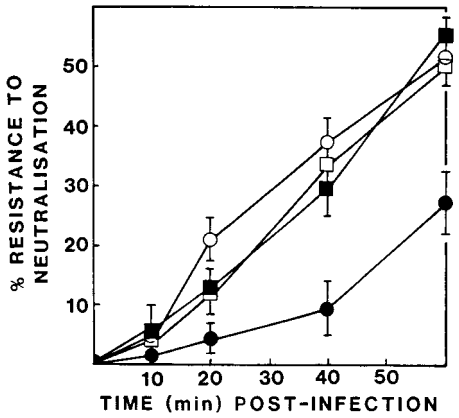

FIG. 3. Penetration of neutralization-resistant variants of RRV T48 into BHK cells. The penetration assay was done as described in Materials and Methods. Each assay was done in triplicate, and the average values are plotted, with error bars showing the standard errors of the means. ●, RRV T48; ○, Tv1; □, Tv3; ■, Tv161.

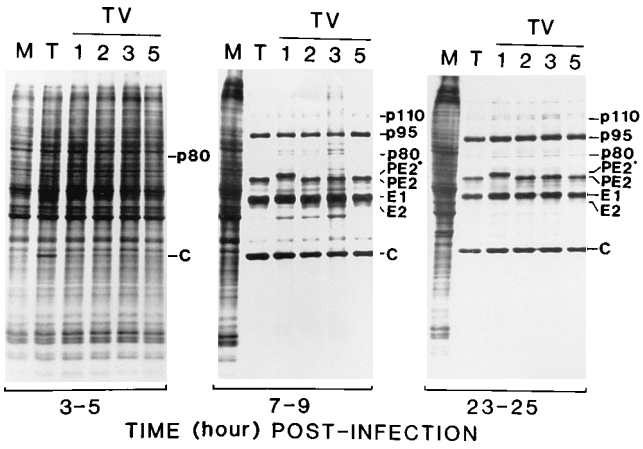

FIG. 5. Viral polypeptide synthesis in BHK cells infected with neutralization-resistant variants of RRV T48. Monolayers of BHK cells were infected with RRV T48 (T), Tv1, Tv2, Tv3, or Tv5 or were mock infected (M) with HBSS. At indicated times the proteins were radiolabeled as described in Materials and Methods, and the cell extracts were electrophoresed on 10 to 20% gradient polyacrylamide gels. The gels were fluorographed and exposed to X-ray films for 6 days. The positions of the viral polypeptides are indicated.

than did the corresponding band in RRV T48-infected cells. Designated pE2*, it had an apparent molecular mass of 64 kDa compared with 62 kDa for RRV T48 pE2. This may be due to additional glycosylation since E2 of these variants had an Asp→Asn mutation (Table 1) which would generate an additional site for asparagine-linked glycosylation. Interestingly, no differences in the migration of intracellular E2 or E3, both of which are produced by the cleavage of pE2, were seen. Whether E2 in purified virions of these variants undergoes additional glycosylation is not known. No differences in shutdown of host protein synthesis between RRV T48- or the variant-infected BHK cells were apparent at any of the time points examined. Thus, the kinetics of viral protein synthesis in

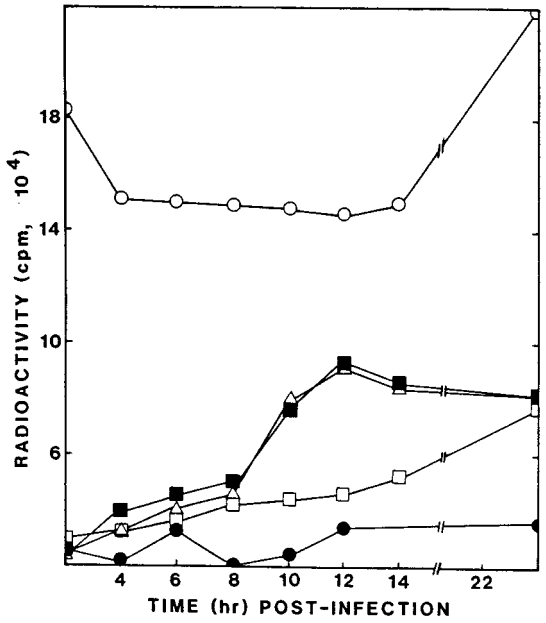

FIG. 4. Actinomycin D-resistant RNA synthesis in BHK cells infected with neutralization-resistant variants of RRV T48. BHK cell monolayers were infected with virus (multiplicity of infection of ~1), or mock infected with HBSS, and incubated in growth medium. At intervals, monolayers were labeled with [5′-^{3}H]uridine as described in Materials and Methods. Infected cells were labeled in the presence of actinomycin D, whereas mock-infected cells were labeled in the presence or absence of actinomycin D. Monolayers were dissociated in SDS, and acid-perceptible radioactivity from ~2×10^5 cells was determined by measuring the level of incorporation of [^{3}H]uridine in newly synthesized RNA. ○, mock-infected cells without actinomycin D; ●, mock-infected cells with actinomycin D; ■, RRV T48-infected cells; △, Tv147-infected cells; □, Tv161-infected cells.

TABLE 1. Virulence of RRV T48 neutralization-resistant variants for week-old mice

Virus	Altered epitope(s)	Amino acid change (position in E2)	LD_{50}[a] (PFU in Vero cells)	AST[a] (days)
RRV T48			0.048 (5)	5.92 (4)
Tv31	a	Thr→Ile (216)	0.056 (3)	6.04 (4)
Tv33	a	Thr→Ile (216)	0.055 (3)	5.38 (3)
Tv35	a	Thr→Ile (216)	0.068 (3)	6.20 (6)
Tv42	a	Thr→Ile (216)	0.075 (4)	5.81 (6)
Tv61	b1	Lys→Gly (234)	0.039 (3)	6.12 (3)
Tv62	b1	Lys→Ile (234)	0.045 (3)	6.43 (3)
Tv63	b1	Lys→Asn (234)	0.043 (3)	5.93 (3)
Tv64	b1	Lys→Glu (234)	0.055 (3)	6.72 (3)
Tv1	b2	Asp→Asn (246)	0.031 (3)	7.08 (5)
Tv2	b2	Arg→Ser (251)	0.206[b] (3)	5.96 (3)
Tv3	b2	Thr→Pro (248)	0.028 (5)	6.60 (3)
Tv5	b2	Asp→Val (246)	0.275[b] (3)	4.84 (3)
Tv51	b2	Asp→Asn (246)	0.050 (3)	6.84 (3)
Tv59	b2	Asp→Asn (246)	0.050 (3)	6.27 (3)
Tv131	a, b1	Thr→Ile (216) Lys→Asn (234)	0.049 (3)	6.00 (3)
Tv147	a, b1	Thr→Ile (216) Lys→Asn (234)	0.053 (3)	5.80 (3)
Tv161	a, b1, b2	Thr→Ile (216) Lys→Asn (234) Asp→Asn (246)	0.079 (3)	8.64[b] (3)

[a] Figures are averages of the number of separate determinations shown in parentheses. The virulence parameters of variants were compared with those of RRV T48 by one-way analysis of variance and the *t* test.

[b] Value is significantly different ($P < 0.05$) from value obtained for RRV T48.

infected BHK cells was consistent with the kinetics of growth and RNA synthesis by RRV T48 and the variants.

Virulence of RRV antigenic variants for mice. For day-old mice, RRV T48 was highly virulent, with an LD_{50} of 0.047 PFU. The infected mice showed severe hind leg paralysis and had an AST of 4.93 days. The LD_{50}s and ASTs obtained with the neutralization-resistant variants of RRV were not significantly different from those obtained with RRV T48 (data not shown). No differences in the time of onset and severity of symptoms in mice infected with the neutralization-resistant variants and RRV T48 were observed.

RRV T48 was also highly virulent for week-old mice; the LD_{50} and AST did not differ significantly between day-old and week-old mice (Table 1). Further, no differences in the time of onset or the severity of symptoms were observed. Most variants yielded differences in LD_{50}s and ASTs that were not significantly different from those obtained with RRV T48, except for Tv2, Tv5, and Tv161 (Table 1). Tv2 and Tv5 had higher LD_{50}s than RRV T48 ($P < 0.05$), although differences in ASTs were insignificant. However, for Tv161-infected mice there was a significant increase ($P < 0.05$) in AST over that seen for mice infected with RRV T48. The LD_{50} of Tv161 was similar to that of RRV T48. None of the variants showed any change in the onset and severity of symptoms compared with RRV T48-infected mice.

Growth of neutralization-resistant variants in week-old mice. In order to examine whether changes in the neutralization domain affect virus tissue tropism and replication in certain tissues, the growth of Tv31 and Tv35 (altered in epitope a), Tv3 and Tv5 (altered in epitope b2), and Tv161 (altered in epitopes a, b1, and b2) was examined in week-old mice for which differences in viral virulence were observed. The tissues selected for examination were hind leg muscles, brain tissue, and blood. Hind leg muscles were chosen because they are the major site of RRV replication in mice (39, 42). Brain tissue was chosen because the extent of RRV replication in neuronal tissue may determine the severity of symptoms and result in death.

Viremia levels for Tv3, Tv5, Tv31, and Tv35 followed the same pattern as the viremia level for RRV T48. All variants exhibited titers similar to those of RRV T48, except for Tv161, which reached a 100-fold-lower titer (Fig. 6; data for Tv31 and Tv35 not shown). No differences in the virus titers for brain tissue and hind leg muscles were observed.

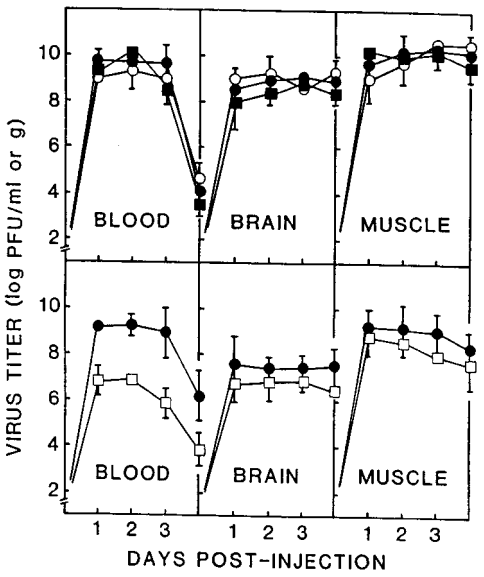

FIG. 6. Growth of neutralization-resistant variants of RRV T48 in mice. Week-old WEHI mice were injected intraperitoneally with ~100 PFU of virus. The mice were killed and tissues were removed at 24-h intervals. The value at each time point is an average of determinations made for three mice. Error bars are shown where the error is greater than 0.2 log units. The amounts of growth of RRV T48, and Tv3, and Tv5 were compared in one experiment (top). In another experiment the amounts of growth of RRV T48 and Tv161 were compared (bottom). ●, RRV T48; ○, Tv3; ■, Tv5; □, Tv161.

DISCUSSION

In our efforts to identify the genetic determinants of RRV virulence, we have studied the effects of single amino acid mutations in the neutralization domain on viral virulence for mice and virus penetration and growth kinetics in BHK cells. Studies with cultured cells showed that variants altered in neutralization epitopes a and/or b1 could not be distinguished from the parent virus. However, epitope b2 variants showed rapid penetration, retarded growth, and delayed RNA and protein synthesis in BHK cells.

An interesting parallel to the biological properties of the variants in BHK cells was seen in mice. Neutralization-resistant variants altered in epitope a or b1 showed no difference in virulence or growth in mice. However, three of the epitope b2 variants (Tv2, Tv5, and Tv161) showed reduced virulence in week-old mice. Furthermore, Tv161 produced lower levels of viremia in mice than did RRV T48.

From the results observed in cultured cells it seems likely that the changes seen in mice did not result from altered recognition of the variants by the immune system. This follows from two considerations. First, the week-old mice used in the present studies are considered immunologically immature (23), and second, any antibody produced in mice would be polyclonal and able to neutralize the antigenic variants effectively. Hence, the changes in biological properties of epitope b2 variants suggest some important function of this epitope besides that of inducing neutralizing antibody.

Variants altered in epitope b2 showed retarded growth in BHK cells. Consistent with this, these variants had smaller amounts of viral RNA and protein synthesized during the early phase of growth. These variants, however, had increased rates of cell penetration. These results suggest that epitope b2 variants may have a lesion in early events of viral infection. The penetration assay used in our experiments actually measures the rate of virus escape from the neutralizing antibody, and thus it may monitor a very early event in the complex virus entry pathway. It may, therefore, be possible that while the mutations in epitope b2 increase the efficiency of an early event in virus entry, a later event in virus infection, such as uncoating, may be slowed down, resulting in initially retarded growth.

It is noteworthy that in our studies, the three RRV variants that showed any attenuation all had a mutation in epitope b2. Variant Tv2 had an Arg→Ser substitution at residue 251, while Tv5 had an Asp→Val substitution at residue 246. However, an Asp→Asn substitution at residue 246 in Tv1 or Tv59 did not result in a change in virulence. The explanation for this probably lies in the nature of the amino acid change. Variant Tv161 had changes in all three epitopes: Thr→Ile at residue 216, Lys→Asn at residue 234, and Asp→Asn at amino acid 246. Since none of the equivalent single amino acid changes altered the virulence of the corresponding variants, it is possible that the attenuation seen for Tv161 was a cumulative effect of multiple mutations.

From our results, it is clear that only changes in epitope b2 altered the virulence of the virus and that even within this

epitope, only certain amino acid substitutions were capable of affecting virulence. Similar observations have been made with VEE virus, for which mutations in only two of the four neutralization epitopes that were identified had an attenuating effect on viral virulence (15). Similarly, for rabies virus only those neutralization-resistant variants that were altered at amino acid 333 of the glycoprotein were attenuated; others that had mutated at nearby amino acid 330, 336, or 338 retained their original virulence (37). It was suggested that arginine 333 was probably within the region of the glycoprotein which interacted with the host cell receptor. For mumps virus also, only one of four neutralization-resistant variants isolated with a single monoclonal antibody had altered virulence; the amino acid changes which took place in the different variants were not identified (20).

Studies with cultured cells and with mice described in this paper suggest a role for epitope b2 in some aspect of viral attachment or entry. This is consistent with studies by Meek et al. (22), who showed that virulent mutants obtained by passaging avirulent RRV NB5092 in mice had changes at amino acids 212, 232, 234, and 251, which were located in neutralization epitopes in E2. As RRV NB5092 was passaged in neonatal mice, in which the immune system is immature, it is unlikely that the mutations were selected as a result of antibody pressure. Since the virulent mutants of RRV NB5092 grew to higher titers in the blood and brains of mice, it may be that mutants which were better able to interact with the cellular receptor were selected. Similarly, serial passage of RRV in chicken embryo fibroblasts selected for virus variants that were altered in their reactions to neutralizing monoclonal antibodies (17). Sequencing of the E1 and the E2 genes established that single amino acid substitutions had occurred at residue 4 or 218 of the E2 protein. Variants that were altered at residue 218 replicated less efficiently in day-old mice than did the parent virus, whereas those altered in residue 4 showed no changes. In light of these results and our findings, we propose that both the receptor binding site and neutralization epitopes of RRV are nearby or in the same domain of E2. Thus, a change in a neutralization epitope could alter the conformation of the receptor binding site, leading to an altered recognition of cellular receptor. This could result in a change in cell or tissue tropism or in an altered rate of viral attachment to or entry into the cell, which may affect the establishment and spread of the infection. Such changes could affect the growth rate and virulence of the virus.

Studies with picornaviruses (3, 8, 32), orthomyxoviruses (44), and rotaviruses (34) suggest that the neutralization antigenic sites of these viruses are involved in virus-cell interaction. Recent studies with a number of alphaviruses also indicate a role of the neutralization domain in early virus-cell interactions, receptor recognition, and pathogenesis (21, 26, 36, 38). Alphavirus entry into vertebrate cells involves the association of the virus with the cell surface, virus binding to a receptor, recruitment to additional receptors, translocation of the complex to the coated pits, and endocytosis (reviewed in reference 18). The penetration assays used here, which measure the kinetics of the acquisition of resistance to the added polyclonal antibody, did not permit an identification of the affected step(s) in the entry pathway. Similar penetration experiments with field isolates of RRV (16a) indicate that mutations in the E1 protein can also influence virus entry rates. This is conceivable since E1 and E2 proteins exist in the virion as a heterodimer (11, 14, 31, 45), and therefore changes in amino acid residues of both E1 and E2 involved in protein-protein interactions could affect virus-cell interactions. Structural analysis of the RRV E1-E2 heterodimer may clarify the three-dimensional structure of the neutralization epitopes. Site-directed mutagenesis of the infectious clone of the virus can then be conducted to clarify the role of individual residues in E1 and E2 in virus entry and virulence.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Health and Medical Research Council of Australia.

We thank R. Cunningham for advice on statistical analysis of data and Eszter Oszko and Rob Ferguson for excellent technical assistance.

REFERENCES

1. **Baric, R. S., D. W. Trent, and R. E. Johnston.** 1981. A Sindbis virus variant with a cell determined latent period. Virology **110:**237–242.
2. **Bassel-Duby, R., D. R. Spriggs, K. L. Tyler, and B. N. Fields.** 1986. Identification of attenuating mutations on the reovirus type 3 S1 double-stranded RNA segment with a rapid sequencing technique. J. Virol. **60:**64–67.
3. **Baxt, B., D. O. Morgan, B. H. Robertson, and C. A. Timpone.** 1984. Epitopes on foot-and-mouth disease virus outer capsid protein VP_1 involved in neutralization and cell attachment. J. Virol. **51:**298–305.
4. **Dalgarno, L., C. M. Rice, and J. H. Strauss.** 1983. Ross River virus 26S RNA: complete nucleotide sequence and deduced sequence of the encoded structural proteins. Virology **129:**170–187.
5. **Davis, N. L., F. J. Fuller, W. G. Dougherty, R. A. Olmsted, and R. E. Johnston.** 1986. A single nucleotide change in the E2 glycoprotein gene of Sindbis virus affects penetration rate in cell culture and virulence in neonatal mice. Proc. Natl. Acad. Sci. USA **83:**6771–6775.
6. **Dietzschold, B., W. H. Wunner, T. J. Wiktor, A. D. Lopes, M. Lafon, C. L. Smith, and H. Koprowski.** 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc. Natl. Acad. Sci. USA **80:**70–74.
7. **Doherty, R. L., E. J. Barrett, B. M. Gorman, and R. H. Whitehead.** 1971. Epidemic polyarthritis in Eastern Australia, 1959–1970. Med. J. Aust. **1:**5–8.
8. **Emini, E. A., S.-Y. Kao, A. J. Lewis, R. Crainic, and E. Wimmer.** 1983. Functional basis of poliovirus neutralization determined with monospecific neutralizing antibodies. J. Virol. **46:**466–474.
9. **Faragher, S. G., I. D. Marshall, and L. Dalgarno.** 1985. Ross River virus genetic variants in Australia and the Pacific Islands. Aust. J. Exp. Biol. Med. Sci. **63:**473–488.
10. **Faragher, S. G., A. D. J. Meek, C. M. Rice, and L. Dalgarno.** 1988. Genome sequence of a mouse-avirulent and a mouse-virulent strain of Ross River virus. Virology **163:**509–526.
11. **Fuller, S. D.** 1987. The T=4 envelope of Sindbis virus is organized by interactions with a complementary T=3 capsid. Cell **48:**923–934.
12. **Gard, G. P., I. D. Marshall, and G. M. Woodroofe.** 1973. Annually recurrent epidemic polyarthritis and Ross River virus activity in a coastal area of New South Wales. II. Mosquitoes, viruses and wildlife. Am. J. Trop. Med. Hyg. **22:**551–560.
13. **Glasgow, G. M., H. M. Killen, P. Liljestrom, B. J. Sheahan, and G. J. A. Atkins.** 1994. Single amino acid change in the E2 spike protein of a virulent strain of Semliki Forest virus attenuates pathogenicity. J. Gen. Virol. **75:** 663–668.
14. **Harrison, S. C.** 1986. Alphavirus structure, p. 21–34. *In* S. Schlesinger and M. J. Schlesinger (ed.), Togaviridae and Flaviviridae. Plenum Press, New York.
15. **Johnson, B. J. B., J. R. Brubaker, J. T. Roehrig, and D. W. Trent.** 1990. Variants of Venezuelan equine encephalitis virus that resist neutralization define a domain of the E2 glycoprotein. Virology **177:**676–683.
16. **Johnston, R. E., and J. F. Smith.** 1988. Selection for accelerated penetration in cell culture coselects for attenuated mutants of Venezuelan equine encephalitis virus. Virology **162:**437–443.

16a. **Kerr, P. J. et al.** Unpublished results.

17. **Kerr, P. J., R. C. Weir, and L. Dalgarno.** 1993. Ross River virus variants selected during passage in chick embryo fibroblasts: serological, genetic and biological changes. Virology **193:**446–449.
18. **Kielian, M., and A. Helenius.** 1986. Entry of alphaviruses, p. 91–159. *In* S. Schlesinger and M. J. Schlesinger (ed.), Togaviridae and Flaviviridae. Plenum Press, New York.
19. **Kinney, R. M., G.-J. Chang, K. R. Tsuchiya, J. M. Sneider, J. T. Roehrig, T. M. Woodward, and D. W. Trent.** 1993. Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5′-noncoding region and the E2 envelope glycoprotein. J. Virol. **67:**1269–1277.
20. **Löve, A., R. Rydbeck, K. Kristensson, C. Örvell, and E. Norrby.** 1985. Hemagglutinin-neuraminidase glycoprotein as a determinant of pathogenicity in mumps virus hamster encephalitis: analysis of mutants selected with monoclonal antibodies. J. Virol. **53:**67–74.
21. **Lustig, S., A. C. Jackson, C. S. Hahn, D. E. Griffin, E. G. Strauss, and J. H. Strauss.** 1988. Molecular basis of Sindbis virus neurovirulence in mice. J. Virol. **62:**2329–2336.

22. **Meek, A. D. J., S. G. Faragher, R. C. Weir, and L. Dalgarno.** 1989. Genetic and phenotypic studies on Ross River virus variants of enhanced virulence selected during mouse passage. Virology **172:**399–407.
23. **Murgita, R. A., and H. Wigzell.** 1981. Regulation of immune functions in the fetus and newborn. Prog. Allergy **29:**54–133.
24. **Newton, S. E., N. J. Short, and L. Dalgarno.** 1981. Bunyamwera virus replication in cultured *Aedes albopictus* (mosquito) cells: establishment of a persistent viral infection. J. Virol. **38:**1015–1024.
25. **Olmsted, R. A., W. J. Meyer, and R. E. Johnston.** 1986. Characterization of Sindbis virus epitopes important for penetration in cell culture and pathogenesis in animals. Virology **148:**245–254.
26. **Pence, D. F., N. L. Davis, and R. E. Johnston.** 1990. Antigenic and genetic characterization of Sindbis virus monoclonal antibody escape mutants which define a pathogenesis domain on glycoprotein E2. Virology **175:**41–49.
27. **Peters, C. J., and J. M. Dalrymple.** 1990. Alphaviruses, p. 713–761. *In* B. N. Fields, D. M. Knipe, et al. (ed.), Virology. Raven Press, Ltd., New York.
28. **Polo, J. M., N. L. Davis, C. M. Rice, H. V. Huang, and R. E. Johnston.** 1988. Molecular analysis of Sindbis virus pathogenesis in neonatal mice by using virus recombinants constructed in vitro. J. Virol. **62:**2124–2133.
29. **Prehaud, C., P. Coulon, F. Lafay, C. Thiers, and A. Flamand.** 1988. Antigenic site II of rabies virus glycoprotein: structure and role in viral virulence. J. Virol. **62:**1–7.
30. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent end points. Am. J. Hyg. **27:**493–497.
31. **Rice, C. M., and J. H. Strauss.** 1982. Association of Sindbis virion glycoproteins and their precursors. J. Mol. Biol. **154:**325–348.
32. **Rossmann, M. G., E. Arnold, J. W. Erickson, E. A. Frankerberger, J. P. Griffith, H.-J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry, and G. Vriend.** 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. Nature (London) **317:**145–153.
33. **Russell, D. L., J. M. Dalrymple, and R. E. Johnston.** 1989. Sindbis virus mutations which coordinately affect glycoprotein processing, penetration, and virulence in mice. J. Virol. **63:**1619–1629.
34. **Sabara, M., J. E. Gilchrist, G. R. Hudson, and L. A. Babiuk.** 1985. Preliminary characterization of an epitope involved in neutralization and cell attachment that is located on the major bovine rotavirus glycoprotein. J. Virol. **53:**58–66.
35. **Santagati, M. G., J. A. Maatta, P. V. Itaranta, A. A. Salmi, and E. Hinkkanen.** 1995. The Semliki Forest virus E2 gene as a virulence determinant. J. Gen. Virol. **76:**47–52.
36. **Schoepp, R. J., and R. E. Johnston.** 1993. Directed mutagenesis of a Sindbis virus pathogenesis site. Virology **193:**149–159.
37. **Seif, I., P. Coulon, P. E. Rollin, and A. Flamand.** 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J. Virol. **53:**926–934.
38. **Strauss, E. G., D. S. Stec, A. L. Schmaljohn, and J. H. Strauss.** 1991. Identification of antigenically important domains in the glycoproteins of Sindbis virus by analysis of antibody escape variants. J. Virol. **65:**4654–4664.
39. **Taylor, W. P., and I. D. Marshall.** 1975. Adaptation studies with Ross River virus: laboratory mice and cell culture. J. Gen. Virol. **28:**59–72.
40. **Tucker, P. C., and D. E. Griffin.** 1991. Mechanism of altered Sindbis virus neurovirulence associated with a single-amino-acid change in the E2 glycoprotein. J. Virol. **65:**1551–1557.
41. **Tyler, K. L., and B. N. Fields.** 1990. Pathogenesis of viral infections, p. 191–239. *In* B. N. Fields, D. M. Knipe, et al. (ed.), Virology. Raven Press, Ltd., New York.
42. **Vrati, S., S. G. Faragher, R. C. Weir, and L. Dalgarno.** 1986. Ross River virus mutant with a deletion in the E2 gene: properties of the virion, virus-specific macromolecule synthesis and attenuation of virulence for mice. Virology **151:**222–232.
43. **Vrati, S., C. A. Fernon, L. Dalgarno, and R. C. Weir.** 1988. Location of a major antigenic site involved in Ross River virus neutralization. Virology **162:**346–353.
44. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature (London) **289:**373–378.
45. **Ziemiecki, A., and H. Garoff.** 1978. Subunit composition of the membrane glycoprotein complex of Semliki Forest virus. J. Mol. Biol. **122:**259–269.