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Sindbis Virus Mutants Selected for Rapid Growth in Cell Culture Display Attenuated Virulence in Animals

Abstract. *Mutants of Sindbis virus were selected for rapid growth in baby hamster kidney (BHK) cell cultures and screened for attenuation of virulence in suckling mice. Comparisons among independently isolated virulent and attenuated strains, as well as a classical reversion analysis, showed that accelerated penetration of BHK cells was correlated with attenuation in vivo. Both phenotypic changes resulted from a reorganization of virion structure as detected by monoclonal antibodies. These results suggest that mutants selected for rapid growth in cell culture may be useful as attenuated vaccines and for studies of the molecular basis of virus pathogenesis.*

It has long been recognized that blind serial passage of viruses in cell culture results in the selection of strains that have decreased pathogenicity in animals and are useful as live virus vaccines. However, the molecular mechanism or mechanisms responsible for attenuation by serial passage are not well understood. One possible basis for this enrichment is that serial passage in cell culture exerts a selective pressure for mutants that efficiently utilize the synthetic processes or structural elements of the tissue culture host. Such mutants presumably would be less efficient in their utilization of the analogous processes in target cells of the natural, animal host, thus restricting their ability to cause overt disease in vivo. Our objective was to test this hypothesis by applying a stringent selective pressure for efficient replication of Sindbis virus (SB) in baby hamster kidney (BHK) cells and by screening the resulting mutants for attenuation in suckling mice. We found that selection for efficient replication in cell culture enriched for mutations affecting one of the SB glycoproteins and that these mutations governed both penetration in vitro and attenuation in animals.

Sindbis virus is a member of the alphavirus genus, a group of viruses carried by arthropods that includes the agents of Eastern, Western, and Venezuelan equine encephalitis (*1*). SB virions consist of a single-stranded RNA of positive polarity (*2*) that is complexed with capsid protein (30,000 molecular weight) to form an icosahedral nucleocapsid (*3*). The nucleocapsid is enclosed within a lipoprotein envelope composed of cellular lipids (*4*) and two virus-specified glycoproteins, E1 and E2 (50,000 and 45,000 molecular weight, respectively) (*3, 5*). Upon subcutaneous inoculation of suckling mice, a single plaque-forming unit (PFU) is sufficient to cause death within 5 days (*6, 7*). The virus replicates locally at the site of injection and gives rise to a viremia that peaks 24 hours after inoculation (*7*). Invasion of the central nervous system occurs by infection of endothelial cells lining the small capillaries of the brain. By 2 days after inoculation, viral antigen is found throughout the brains of infected animals, and high virus titers are sustained until death. Mortality is invariably 100 percent.

Since viruses are obligate intracellular parasites, their replication depends heavily on the interaction of virus-specified products with structural and enzymatic elements of the host. We reasoned (i) that the efficiency of these types of interactions between virus and cell could be affected by mutation, (ii) that a significant increase in efficiency would lead to a decrease in the time required for the completion of the entire replicative cycle, and (iii) that mutants exhibiting improved efficiency would be among those progeny virions released from infected cells soon after the end of the latent period. Accordingly, SB was grown in BHK cells, and virus was harvested 5.5 hours after infection, which is 1 to 2 hours after the end of the normal latent period (*8*). Undiluted virions were then used as inoculum for a second passage, and the rapid-growth selection was continued in this manner for 12 serial passages (*9*). Thirty putative mutants were cloned by plaque purification from each of passages 4 to 12, and over 100 of these were tested for virulence in suckling mice. The isolates fell into three virulence categories: those which resembled wild-type, infected mice having a mean survival time of 5 to 6 days and 100 percent mortality; a marginally attenuated group, infected mice having a mean survival time of 8 to 9 days and an occasional survivor; and an attenuated group characterized by mortality rates ranging from 90 to 0 percent with mean survival times from 10 to 14 days (Fig. 1).

Attenuated isolates were found in passage 4, and the proportion of attenuated strains increased at each round of rapid growth selection from 7 percent in passage 4 to 70 percent in passage 12. In contrast to mutants selected for rapid growth, our wild-type SB stock had been maintained by passage with an 18-hour growth cycle. Of 30 independent plaque-purified isolates from wild-type stock, all showed 100 percent mortality and a mean survival time of 4 to 5 days. Selection for rapid growth therefore increased the number of mutants that displayed reduced virulence.

A prototype attenuated mutant from passage 11 was characterized further. This mutant had a reduced latent period in BHK cells compared to SB and was designated SB-RL. This mutant was indistinguishable from its wild-type parent on the basis of the following criteria: temperature sensitivity; *p*H optima for hemagglutination or cell-to-cell fusion from within or without; attachment to BHK cells; ability to induce homologous interference; induction of interferon in vitro or sensitivity to interferon; ability to produce defective-interfering particles after passage at high multiplicities of infection; buoyant density in potassium tartrate gradients; sedimentation velocity in sucrose; mobility in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the virion proteins; and two-dimensional analysis of ribonuclease T1 oligonucleotides derived from the genomic RNA's (*10*). However, the reduction in the SB-RL latent period resulted from two aspects of SB-RL rep-

lication in vitro, which indicated that our selection for efficient interaction between virus and cell had been effective. First, the maximum rate of RNA synthesis in SB-RL–infected cells was twice that of SB (*10*). We and others have suggested that SB RNA synthesis requires one or more host factors (*11*), and selection for rapid growth might be expected to select for efficient utilization of such factors. The second indication of efficient interaction between virus and cell was that SB-RL exhibited an accelerated penetration rate in BHK cells compared to SB (*10*). SB-RL was also distinguishable from SB on the basis of its smaller plaque size.

Our next objective was to determine which, if any, of these characteristic differences between SB and the prototype attenuated strain was associated with reduced virulence in vivo. First, we analyzed a number of attenuated and virulent isolates from the original rapid growth series in an attempt to correlate attenuation in vivo with one or more of those characteristics that distinguished SB from SB-RL in tissue culture. Second, several virulent revertants of SB-RL were isolated from the brains of moribund, SB-RL–infected mice and used in a classical reversion analysis. Both analyses showed that neither plaque size nor enhanced RNA synthesis was correlated with pathogenicity. We found several small plaque isolates that were virulent as well as several large plaque attenuated variants. All isolates examined—whether virulent, marginally attenuated, attenuated, or virulent revertants of SB-RL—exhibited maximal rates of viral RNA synthesis that were two to six times that of wild type (data not shown). However, a strong correlation was observed between rapid penetration of BHK cells (as measured by rate of escape from neutralization by extracellular antibody) and attenuation in suckling mice (Fig. 2). Moreover, strains that had reverted from attenuated to wild-type virulence also reverted with respect to penetration.

An alteration in one of the surface glycoproteins of the virion could affect both interaction with BHK cells in culture and tissue tropism in infected animals. Although a detectable alteration in the viral glycoproteins was not evident by SDS-PAGE, polyclonal antiserum to SB-RL clearly neutralized SB-RL and other attenuated isolates more efficiently than SB and other virulent strains (*8*). In addition, reversion to wild-type virulence was coincident with reversion to immunologic reactivity characteristic of SB. To investigate further the antigenic distinction between virulent and attenuated strains, we generated several hybridoma cell lines producing monoclonal antibody to the attenuated prototype, SB-RL. Two of these (monoclonal antibodies 6 and 13) distinguished attenuated from virulent strains on the basis of 80 percent neutralization end point determinations (Fig. 3). These did not neutralize the four virulent revertant strains or another virulent Sindbis strain, SB^{dal} (*12*). When tested against six virulent and nine attenuated clones from the passage series, each of the virulent clones resembled SB and each of the attenuated isolates resembled SB-RL.

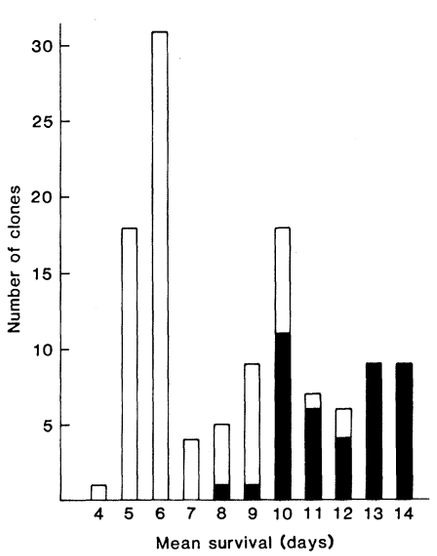

Fig. 1. Pathogenicity of plaque-purified strains isolated from passages 4 to 12 of the rapid growth selection series. Stocks of each isolate were grown and titered on BHK cells. Litters of 2-day-old mice (ICR-L^+) (*20*) were inoculated subcutaneously with 25 to 50 PFU per animal (ten mice per litter per isolate). The mean survival time characteristic of each isolate was determined by using an observation period of 14 days. The shaded bars denote strains having less than 100 percent mortality.

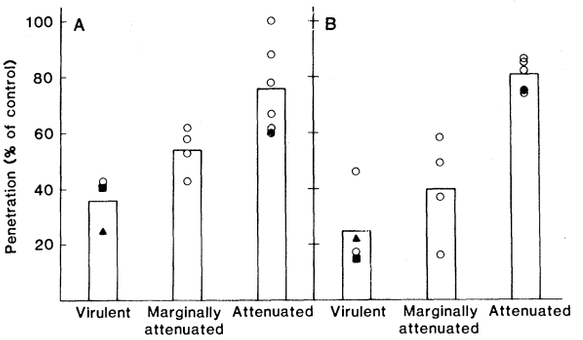

Fig. 2. Penetration of BHK cells. In two separate experiments (A and B), BHK cells were infected with 100 PFU per plate of either SB (■), SB-RL (●), one of the virulent revertants (▲), or one of 20 different isolates from the three pathogenesis categories (○). Virus was allowed to adsorb and penetrate at 37°C for 15 minutes, at which time the inoculum was removed and the monolayers washed with phosphate-buffered saline. Rabbit antibody to SB, which effectively neutralized each of the isolates used in these experiments, was added to each culture for 30 minutes at 37°C to neutralize any extracellular virus. The plates were washed and overlayed for visualization of plaques. The open bars represent the mean values for each of the three pathogenesis categories. A significant difference (Student's *t*-test, $P < 0.005$) was observed between the attenuated isolates (including SB-RL) and virulent isolates (including SB and the virulent revertants).

The protein specificity of monoclonal antibodies 6 and 13 was determined by radioimmune precipitation of SB-RL–infected cell lysates (Fig. 4), and both recognized an epitope on glycoprotein E2. However, the epitope recognized by monoclonal antibodies 6 and 13 in neutralization assays with SB-RL also was present on wild-type E2, as shown by

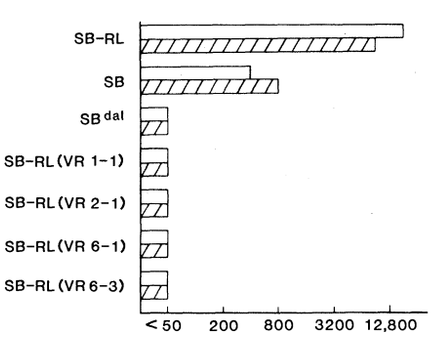

Fig. 3. Neutralizing activity of two monoclonal antibodies to SB-RL. Monoclonal antibodies were prepared by polyethylene glycol–mediated fusion of spleen cells from BALB/c-Cum mice (Cumberland Farms) immunized with SB-RL and S194/5.XX0.BU.1 myeloma cells (American Type Culture Collection) in the presence of HAT medium (medium containing hypoxanthine, aminopterin, and thymidine) (*21*). Twelve to 16 days after fusion, wells containing hybrid cells were screened by an enzyme-linked immunosorbent assay (*22*) against SB-RL. The positive cultures were expanded and cloned three times by limiting dilution. Antibody stocks were obtained by injecting the hybridomas into BALB/c-Cum mice previously treated with pristane for production of ascites (*21*). The neutralization indices of monoclonal antibodies 6 and 13 (open and hatched bars, respectively) were measured in a plaque-reduction neutralization assay in the presence of 5 percent guinea pig complement (*23*). Neutralization of SB-RL, SB, the four virulent revertant (VR) isolates, and another virulent Sindbis strain, SB^{dal} (*12*), is indicated as the reciprocal of the highest antibody dilution at which plaque reduction greater than or equal to 80 percent was observed.

specific immune precipitation of SB E2 by monoclonal antibodies 6 and 13 and by the equivalent reactivity of these antibodies to disrupted SB and SB-RL in solid-phase immunoassays (*13*). Therefore, we propose that the mutation leading to attenuation of SB resulted in a reorientation of glycoprotein E2 such that the epitope recognized by monoclonal antibodies 6 and 13 became more accessible to neutralizing antibody. We do not yet know whether the increased exposure of this epitope is responsible for the phenotype of SB-RL or, alternatively, if the reorientation of E2 reflects a primary mutation in another portion of E2, in E1, or perhaps in the capsid protein with which the glycoproteins presumably interact (*14*).

The relation between accelerated penetration, monoclonal reactivity, and attenuation was examined further by applying a selective pressure for rapid penetration under conditions that would not be expected to affect other aspects of the replication cycle (*15*). Wild-type SB was allowed to adsorb to BHK cells for 1 hour at 4°C, a temperature at which penetration does not occur. The cultures were warmed to 37°C for 1 minute, washed with phosphate-buffered saline, and treated with trypsin to remove any virions that remained extracellular. The cells were suspended in medium, and the progeny were harvested after 18 hours. After five such passages, a rapidly penetrating mutant was isolated by plaque purification. This mutant was preferentially neutralized with monoclonal antibody 6 to the same degree as SB-RL and was highly attenuated in suckling mice.

These results suggest that selection for efficient replication in cultured cells is one of the operable selective pressures in serial blind passage regimens used to generate attenuated virus strains. In the SB model system, the process of attenuation was accelerated by increasing the selective pressure for efficiency with a stringent requirement for rapid growth. Of particular interest was the strong correlation between attenuation in vivo on one hand and efficient penetration of cultured cells and preferential neutralization by monoclonal antibodies 6 and 13 on the other. Mutation in or reorientation of glycoprotein E2 (or both) significantly affected the penetration of the virus with a concomitant decrease in its capacity to cause disease in animals. This is in keeping with other findings in that mutation of proteins exposed at the surface of virions profoundly affects pathogenesis in reovirus (*16*), influenza (*17*), rabies (*18*), and Venezuelan equine encephalitis (*19*). Since many of these same proteins are doubtless involved in interactions between virus and cell that lead to successful penetration, it is not surprising that mutations affecting penetration in cell culture might also alter tissue tropism and pathogenicity in vivo. Therefore, selection for rapid growth, and in particular selection for rapid penetration, may be generally applicable in other virus systems for the isolation of mutants characterized by rearrangements at the virion surface and altered pathogenicity. Such mutants will facilitate studies of the molecular basis of virus pathogenesis, and some may prove useful as live virus vaccines.

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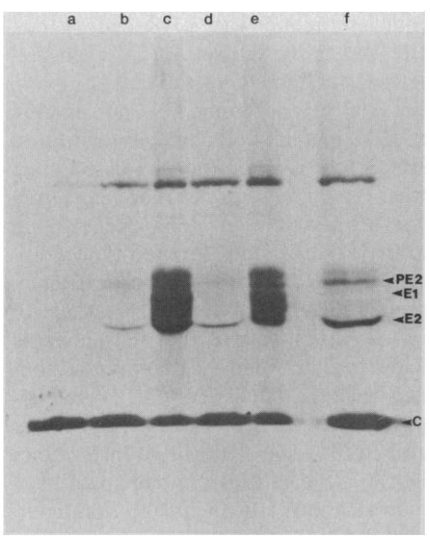

Fig. 4. Radioimmune precipitation of SB-RL–infected cell lysates by monoclonal antibodies 6 and 13. BHK cells were infected with SB-RL (50 to 100 PFU per cell). After a 1-hour adsorption period, the inoculum was replaced with methionine-free Eagle minimum essential medium containing 5 percent donor calf serum (MA Bioproducts) and 5 μg of actinomycin D per milliliter. Cells were treated from 4 to 8 hours after infection with 20 μCi of [^{35}S]methionine per milliliter (New England Nuclear) to label viral structural proteins. The monolayers were washed three times with cold phosphate-buffered saline, and the cells were collected by centrifugation. The cell pellet was solubilized at 4°C for 5 minutes in lysis buffer [0.02*M* tris-HCl (*p*H 7.5), 0.15*M* NaCl, 1 percent Triton X-100, 0.5 percent sodium desoxycholate, and 10 μ*M* phenylmethylsulfonyl fluoride]. The lysate was centrifuged in a Microfuge for 2 minutes, and the supernatant (100 μl per sample) was combined with the appropriate antibody (50 μl) and incubated overnight at 4°C. Pansorbin (Calbiochem) (50 μl) was added to each sample and incubated for 30 minutes at room temperature. The immune complexes were pelleted in a Microfuge and washed five times with lysis buffer. Final pellets were boiled for 5 minutes in electrophoresis sample buffer [0.0625*M* tris-HCl (*p*H 6.8), 10 percent glycerol, 2 percent SDS, 5 percent β-mercaptoethanol] and centrifuged for 1 minute. The supernatants were analyzed by SDS-PAGE (*24*) and autoradiography (*25*). The following antibodies were used: lane a, normal mouse serum; lanes b and d, monoclonal antibodies 6 and 13, respectively; lanes c and e, SB and SB-RL–specific mouse hyperimmune ascitic fluid, respectively; lane f, a known E2-specific monoclonal antibody (*26*). Capsid protein bound nonspecifically to the Pansorbin; the 100K protein, an unprocessed fusion product of PE2 and E1 (*14*), was precipitated specifically by the antibody preparations used in lanes b to f.

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Voltage-Dependent Calcium Channels from *Paramecium* Cilia Incorporated into Planar Lipid Bilayers

Abstract. *Two different divalent cation–selective channels from* Paramecium *cilia were incorporated into planar lipid bilayers. Both channels were much more permeable to divalent than univalent cations, and one of them discriminated significantly among the divalent cations. The selectivity and voltage dependence of the latter channel are comparable to those of voltage-dependent calcium channels found in a variety of cells. A combined biochemical, biophysical, and genetic study of calcium channels is now possible.*

Although Ca^{2+} currents mediate a large variety of cellular processes, Ca^{2+} channels have been difficult to study in cell membranes because of problems in controlling transmembrane voltage, in varying ionic composition, and in isolating Ca^{2+} currents (*1*). These cell-related problems can be avoided by incorporating Ca^{2+} channels into a planar lipid bilayer. We used vesicles made from *Paramecium* ciliary membranes because these cilia are an excellent source of Ca^{2+} channels that are well characterized electrophysiologically (*2*) and easily modified genetically and biochemically (*3*, *4*). We found that *Paramecium* ciliary membranes contain two types of channels that are divalent cation–selective. Although both channels are virtually impermeable to monovalent cations, the channels differ in conductance, divalent cation selectivity, and voltage dependence. The larger [30-picosiemens (pS)] channel selects poorly among divalent cations and is only weakly voltage-dependent. The smaller (1.5- to 2-pS) channel is very selective (Ca^{2+} and Ba^{2+} can be current carriers, but Mg^{2+} is virtually impermeant) and is strongly voltage-dependent (an *e*-fold change in membrane conductance for an ~9-mV change in membrane potential). The larger channel may be related to the mechanoreceptor of *Paramecium* (*5*) and the smaller channel is probably responsible for the Ca^{2+} current measured in the intact *Paramecium*. [A Ca^{2+}-activated K^+ channel has probably also been identified in ciliary membranes by incorporation into planar bilayers (*6*)].

Ciliary membrane vesicles were prepared as described by Adoutte *et al.* (*7*), except that 1 m*M* KCl was added to all solutions after deciliation. Experiments were done with six different preparations of vesicles; fresh or frozen vesicles yielded the same results. Membranes made from asolectin (90 percent) and cerebroside (10 percent) or from phosphatidylethanolamine (75 percent), phosphatidylserine (15 percent), and cerebroside (10 percent) were formed by painting a solution of lipid in decane across a 50- to 100-μm hole in a Teflon sheet that bisected a Lucite chamber (*8*). Cholesterol was added to the lipid solution in some experiments, but had no detectable systematic effects. Vesicles were always added to the *cis* compartment. All experiments were done at room temperature under voltage-clamp conditions. Values for the holding potentials refer to the potential of the *cis* compartment with respect to the *trans* compartment, which was held at virtual ground. Membrane currents were amplified, converted to

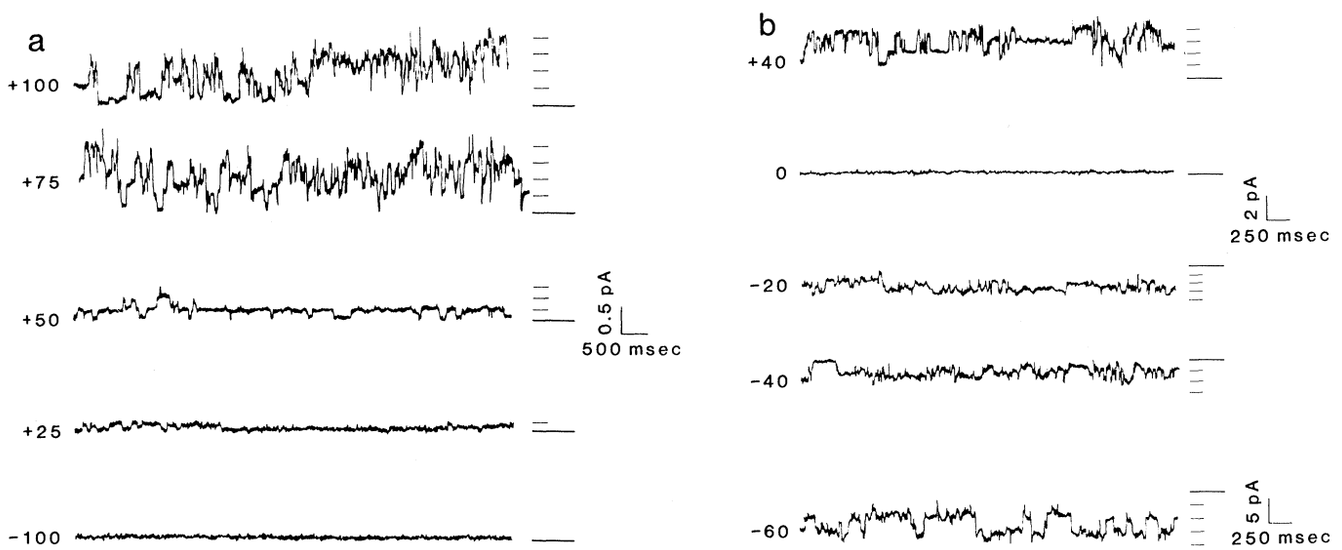

Fig. 1. Single-channel currents from *Paramecium* ciliary membrane vesicles incorporated into a planar bilayer. Long horizontal lines to the right of current traces indicate the zero current level and short lines indicate single-current steps; bare bilayer current has been subtracted in all records. Numbers on the left indicate the holding potential of the *cis* compartment with respect to the *trans* compartment, which is held at virtual ground. Upward deflections from the zero current level represent positive current from the *cis* to *trans* compartment. (a) Records from an experiment in which only the small channel was observed with 50 m*M* $BaCl_2$ (*cis*) and 50 m*M* $MgCl_2$ and 0.1 m*M* $BaCl_2$ (*trans*). Single-channel conductance is 1.6 pS. During the experiment, potential steps were randomized so that "off" periods cannot be interpreted as "no-channel" periods. This membrane contains four active channels. The probability of observing an open channel increases with more positive potentials (see Fig. 3). Records were filtered at 60 Hz. (b) Records from an experiment in which only the larger channel was observed with 10 m*M* $BaCl_2$ (*cis*) and 10 m*M* $MgCl_2$ and 0.1 m*M* $BaCl_2$ (*trans*). Single-channel conductance is 29 pS. This membrane contains four active channels that are on most of the time, independent of voltage. Records were filtered at 150 Hz.