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Antibody-Selected Variation and Reversion in Sindbis Virus Neutralization Epitopes

DAVID S. STEC,[1] ANNA WADDELL,[1] CONNIE S. SCHMALJOHN,[2] GERALD A. COLE,[1] AND ALAN L. SCHMALJOHN[1]*

Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland 21201,[1] and Virology Division, U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Frederick, Maryland 21701[2]

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Sindbis virus variants evidencing a complex and bidirectional tendency toward spontaneous antigenic change were isolated and characterized. Variants were selected on the basis of their escape from neutralization by individual monoclonal antibodies to either of the two envelope glycoproteins, E2 and E1. Multisite variants, including one altered in three neutralization sites, were obtained by selecting mutants consecutively in the presence of different neutralizing monoclonal antibodies. Two phenotypic revertants, each of which reacquired prototype antigenicity, were back-selected on the basis of their reactivity with a neutralizing monoclonal antibody. An incidental oligonucleotide marker distinguished these and the variant from which they arose from parental Sindbis virus and other mutants, thereby confirming that the revertants were true progeny of the antigenic variant. Prototype Sindbis virus and variants derived from it were compared on the basis of their reactivities with each of a panel of monoclonal antibodies; patterns revealed a minimum of five independently mutable Sindbis virus neutralization epitopes, segregating as three antigenic sites (two E2 and one E1).

Neutralizing antibodies (N-Abs) figure prominently in protective immune responses to alphaviruses, and the appearance of neutralizing activity in serum generally is associated with recovery from infection and resistance to reinfection (3). Through passive transfer of individual neutralizing monoclonal antibodies (N-mAbs) to nonimmune mice, it has been demonstrated (2, 12, 17) that circulating N-mAbs of the appropriate virus specificity are sufficient to prevent lethal encephalitis caused by Sindbis virus (SIN), Venezuelan equine encephalitis virus, or Semliki Forest virus.

Alphaviruses share antigenic, biologic, and structural features which suggest a common progenitor (1, 5, 8). However, their neutralization epitopes (N-Eps) tend to be polymorphic in that each virus elicits antibodies which neutralize only the immunizing species and closely related ones assigned to the same serological complex (11). Variation in N-Eps is demonstrable not only within each complex but also among naturally occurring strains of a single alphavirus species (15, 16). Although knowledge has accrued on the molecular biology of these viruses, including amino acid sequences of the structural proteins of SIN, Semliki Forest virus, and Ross River virus (5, 8, 14), the molecular bases for alphavirus antigenic changes are unknown, the sites bound by N-Abs have not been clearly defined, and mechanisms of neutralization remain speculative.

To investigate relevant SIN antigens in greater detail and explore mechanisms of antibody-mediated protection, we prepared and characterized mAbs, each reactive with one of the two SIN envelope glycoproteins, E1 and E2. These proteins are similar in size (52,000 and 50,000 daltons, respectively), are incorporated into virions during viral budding at plasma membranes, and together, in apparently equimolar ratios, comprise the virion spikes (8). Consonant with the reported reactivities of antisera to individual SIN proteins (6), we found that most N-mAbs bound to E2. A single E1-specific mAb, functionally similar to one described by Chanas et al. (4), also neutralized SIN, thereby confirming that N-Eps do not reside exclusively on E2.

In studies described below, antigenic variants of SIN—selected for resistance to neutralization by individual N-mAbs—were isolated and characterized. Consecutive selection with appropriate N-mAbs resulted in multisite SIN variants including one having alterations in three independently mutable neutralization sites. To explore the possibility of antigenic reversion and to facilitate future studies on the molecular bases of SIN antigenic change, we selected from variant virus populations revertants that regained ancestral antigenic properties.

MATERIALS AND METHODS

Virus growth and purification. SIN strain AR339 (6, 20) was obtained from Joel M. Dalrymple. A suckling mouse brain suspension, containing approximately 4×10^8 PFU/ml and stored in working samples at −70°C, served as a virus seed stock for initial studies with mAbs including assays of their biological properties and antigenic specificities (18). To ensure a homogeneous parental virus population for the subsequent selection of SIN antigenic variants, the brain-passaged virus was subjected to three successive plaque isolations from (agarose-overlaid) Vero cell monolayers. No antigenic differences between the plaque-purified and original (presumably more heterogeneous) brain-passaged SIN have been observed.

To obtain purified virus, SIN was propagated in Vero cells, precipitated from cell culture supernatants with polyethylene glycol, and centrifuged to equilibrium in 15 to 33% (wt/wt) potassium tartrate gradients as previously described (18). In accordance with the reported absence of host proteins in alphavirus particles (8), stained polyacrylamide gels indicated that, relative to the three SIN structural proteins, cell or serum proteins were generally only minor components of the total protein present in each purified SIN preparation.

mAbs. The preparation and characterization of SIN-

* Corresponding author.

specific mAbs were described previously (17, 18). An additional N-mAb (no. 51) was obtained more recently by fusing BALB/c immune spleen cells with the Sp2/0 mouse myeloma cell line. Ascitic fluids (each containing a minimum of 1 mg of mAb per ml) were prepared in BALB/c mice, stored in working samples at −20°C, and used in the following experiments. For the neutralization assays, mAbs purified by chromatography on protein A-Sepharose (Pharmacia Fine Chemicals) were used.

Selection and isolation of antigenic variants. Antigenic variants were isolated from plaque-purified SIN AR339 by methods similar to those described by Gerhard and Kendal (9) for influenza virus, except that, to make the selection conditions more stringent, guinea pig serum (Cedarlane) was included as a source of complement. In brief, a 10-fold dilution series of SIN AR339 was made in RPMI 1640 medium which contained the selecting N-mAb (10% [vol/vol] as ascitic fluid), 5% (vol/vol) complement, and 10% (vol/vol) fetal bovine serum (Dutchland). Mixtures were incubated for 1 h at 37°C, and then six replicate samples (0.1 ml) of each were added to 96-well tissue culture plates containing 2×10^4 Vero cells per well. Plates were incubated at 37°C in 5% CO_2 for 4 to 5 days and inspected daily for cytopathology. Supernatants from individual wells exhibiting cytopathology were treated again with complement and the same N-mAb (as described above) and plaqued on Vero cell monolayers. Well-isolated plaques were selected, and another plaque assay was performed on Vero cells under nonselecting conditions (in the absence of N-mAb and complement); viruses were passaged once and frozen in working samples at −70°C to provide a stock of each. The isolation frequency of each antigenic variant was estimated from the ratio of the 50% tissue culture infective dose observed in the presence of mAb to that seen under nonselecting conditions (9, 21). Each variant was designated by a "v" followed by the number of the N-mAb used for its selection (e.g., SINv23 was selected for resistance to N-mAb no. 23).

Consecutive variants. Certain SIN variants were subjected to another round of selection and plaque purification, as described above, in the presence of individual N-mAbs with which they had retained reactivity. These consecutive variants were designated by the mAbs used as well as the order of selection (e.g., SINv23/50 arose from SINv23 after selection with N-mAb no. 50).

Phenotypic revertants. Revertants were recovered from populations of SIN antigenic variants by sequential enrichment with an appropriate solid-phase immunoadsorbent. In brief, the selecting mAb (filter sterilized and adjusted to approximately 10 μg/ml) was adsorbed to the growth area of a 25-cm^2 culture flask. Unadsorbed mAb was removed; to deter subsequent nonspecific adsorption, each flask was rinsed with fetal bovine serum. To the flask was added a population (>10^6 PFU) of plaque-purified SIN variant which was unreactive with the solid-phase mAb but which putatively contained a small fraction of antigenic phenotype revertants. After incubation, unbound virus was rinsed from the flask, and Vero cells were added. After cytopathic effect developed, the supernatant from the flask was collected and subjected to another round of solid-phase enrichment as described above. Supernatants from each cycle of enrichment were tested by indirect immunofluorescence and by enzyme-linked immunosorbent assay (ELISA) for the presence of antigenic phenotype revertants. Ultimately, revertants were plaque purified, propagated, and further characterized.

ELISA. As previously described, gradient-purified SIN was affixed to polystyrene microtiter plates, and a two-step procedure (mAb followed by peroxidase-conjugated goat anti-mouse immunoglobulin) was used to assess the binding of each mAb to the solid-phase antigen (18). As a minor change from previous assays, plates were coated with bovine serum albumin (0.5% [wt/vol] in phosphate-buffered saline) rather than fetal bovine serum to deter nonspecific interactions.

To evaluate reactivities between mAbs and SIN antigenic variants, twofold dilutions of purified virus in phosphate-buffered saline were added (50-μl samples) to microtiter wells in amounts ranging from 1 to 0.03 μg per well. Plates were dried overnight to facilitate attachment and effect partial disruption of SIN (18) and then blocked with bovine serum albumin. Individual mAbs, each at a single concentration (>10 μg/ml), were tested by ELISA against duplicate wells of each antigen dilution.

To screen for the presence of antigenic phenotype revertants, a modified "antigen-capture" ELISA was used. In brief, microtiter wells were coated with mAb (approximately 10 μg/ml), washed with phosphate-buffered saline–bovine serum albumin, and overlaid with dilutions of virus-containing cell supernatant. Unbound virus was rinsed away, and virus captured on the solid phase was detected by adding a saturating concentration of an appropriate peroxidase-labeled mAb (18), washing, and adding enzyme substrate.

Neutralization assays. Plaque-reduction neutralization assays were performed as described previously (18), but without exogenous complement. In brief, virus was preincubated with antibody for 45 min, and residual infectivity was measured by plaque assay on Vero cells; the percent plaque reduction was calculated relative to medium controls, which contained approximately 100 PFU per 0.1 ml.

RNA oligonucleotide analyses. ^{32}P-labeled intracellular RNAs from SIN and from selected SIN variants were prepared, and the 26S subgenomic mRNAs were separated from genomic (42S) RNA by velocity sedimentation in 5 to 30% sucrose gradients. Oligonucleotide maps (fingerprints) were prepared by digesting RNA with T_1 RNase and then subjecting the fragments to two-dimensional electrophoresis as described previously (19). To obtain RNA sequences of specific oligonucleotides, SIN virion RNA was digested with T_1, the 5′ ends of the oligonucleotides were labeled with [γ-^{32}P]ATP, and RNA fingerprints were prepared (19). Oligonucleotides were cut from frozen gels, extracted, subjected to base-specific enzyme cleavage (P-L Biochemicals), and analyzed by gel electrophoresis (7).

RESULTS

Characterization of antigenic variants. Antigenic variants of SIN, selected from parental virus on the basis of their resistance to neutralization by individual mAbs, were propagated and purified from infected cell culture supernatants. The reactivities between mAbs and SIN variants (Fig. 1 and Table 1) were evaluated by ELISA. Each variant was unreactive with the N-mAb used for its selection. Most other N-mAbs either lost all detectable reactivity and therefore resembled the selecting mAb or, alternatively, reacted as well with the antigenic variant as with parental SIN. In a few instances (e.g., mAb no. 49 on SINv50) binding remained detectable, but was reduced by more than 50%. Nonneutralizing mAbs, including those specific for capsid or for E1 sites shown in previous competitive binding assays to differ from the E1 N-Ep (18), retained full reactivity with all SIN variants.

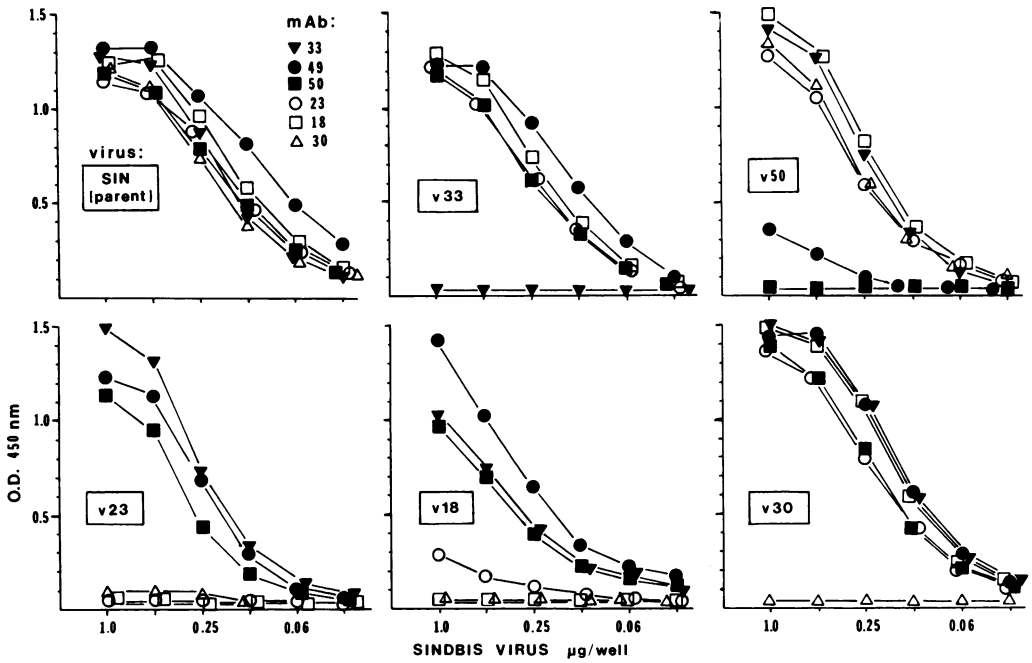

FIG. 1. Characterization of SIN variants by ELISA. Each virus was affixed, in replicate twofold dilutions, to polystyrene wells and allowed to react with constant concentrations (≥10 µg/ml) of individual mAbs. A different virus (as indicated) is shown in each panel, and a key to symbols used for different N-mAbs is given in the top left panel.

Three discrete E2 N-Eps (defined by mAbs no. 23, 18, 30, and 51) changed independently in some cases and concomitantly in others (Table 1); since the molecular relationships among these N-Eps are unknown, we conservatively assigned them to a single complex antigenic site. A second and apparently independent E2 site was defined by N-mAbs no. 49 and 50. A third mutable site was defined by the E1-specific N-mAb, no. 33.

SIN antigenic variants arose spontaneously at frequencies estimated between $10^{-3.5}$ and 10^{-5}, consistent with mutation rates reported for other RNA viruses (9, 10, 21). At the population level, variants appeared quite stable; unless revertants were intentionally selected (see below), each epitope variant retained its phenotype throughout subsequent steps of propagation, purification, or secondary selection.

TABLE 1. Antigenic phenotypes of SIN variants and revertants

SIN variant and selecting mAb	Reactivity[a] with the following mAb:								
	Anti-E2						Anti-E1		Anti-C 3
	50	49	30	51	18	23	33	Others[b]	
SIN	+	+	+	+	+	+	+	+	+
SINv50	0	θ	+	+	+	+	+	+	+
SINv30	+	+	0	0	+	+	+	+	+
SINv51	+	+	0	0	0	0	+	+	+
SINv18	+	+	0	0	0	θ	+	+	+
SINv23	+	+	0	0	0	0	+	+	+
SINv33	+	+	+	+	+	+	0	+	+
SINv50/23	0	0	0	0	0	0	+	+	+
SINv30/23	+	+	0	ND	0	0	+	+	+
SINv23/50	0	0	0	0	0	0	+	+	+
SINv33/50/23[c]	0	0	0	0	0	0	0	+	+
SINv23-R23[d]	+	+	+	+	+	+	+	+	+
SINv23/50-R23[d]	0	θ	0	θ	0	+	+	+	+

[a] ELISA reactivity determined as described in the legend to Fig. 1 and scored, relative to SIN, as positive (+), negative (0), or detectable but reduced by more than 50% (θ). ND, Not determined.

[b] Representative mAbs to each of four additional and topologically distinct E1 sites not implicated in neutralization (18).

[c] Multisite variant obtained by consecutive selection with N-mAbs no. 33, 50, and 23.

[d] Revertants obtained by back-selection with N-mAb no. 23.

Consecutive variants. It was possible to obtain greater divergence from parental SIN by treating viruses consecutively with N-mAbs of different site specificities. Thus, N-mAb no. 50 was used to select, from SINv23, a double mutant (SINv23/50) which lost reactivity with all E2-specific mAbs in our panel (Table 1). A three-step variant, SINv33/50/23, was obtained by consecutive selection with N-mAbs no. 33 (E1), 50 (E2), and 23 (E2). The latter virus lost reactivity with all of our 10 N-mAbs (only 7 are shown) but retained reactivity with nonneutralizing mAbs including those previously shown to mediate protection in vivo (18).

Neutralization of variant viruses. In agreement with the above binding assays, variant viruses were not neutralized by the N-mAbs used in their selection or by other N-mAbs with which they were unreactive in the ELISA. On the other hand, hyperimmune anti-SIN serum neutralized even the three-step variant, which was refractory to neutralization by N-mAbs to the three sites defined above (Table 2). Whether rabbit or mouse hyperimmune anti-SIN serum was used, SINv33/50/23 was neutralized but required 5- to 10-fold higher serum concentrations to effect comparable reductions in plaque numbers.

Phenotypic revertants. Lacking any known biological marker (e.g., cell tropism, temperature sensitivity, plaque size) which paralleled SIN antigenic change, we selected virus revertants solely on the basis of their antigenic phenotypes. Using the procedures detailed in Materials and Methods, we obtained two revertants that regained the ability to react with N-mAb no. 23. The one referred to as SINv23-

TABLE 2. Neutralization of SIN and three-site variant by mAbs or immune sera

Antibody[a]	% Plaque reduction	
	SIN	SINv33/50/23
33 (anti-E1)	94	0[b]
50 (anti-E2)	84	0
49 (anti-E2)	100	0
23 (anti-E2)	85	0
18 (anti-E2)	89	0
3 (anti-C)	0	0
Rabbit anti-SIN 1:10	100	100
1:100	97	76
1:1,000	73	24
Mouse anti-SIN 1:10	100	100
1:100	93	60
1:1,000	67	0
1:10,000	36	0

[a] N-mAbs were each tested at 10 μg/ml; when diluted and tested against SIN, their 50% plaque reduction neutralization titer endpoints were approximately 0.03 μg/ml (mAbs no. 49 and 50), 0.1 μg/ml (no. 23), and 1.0 μg/ml (nos. 18 and 33). Sera from rabbit or C57BL/6 mice (immunized with purified SIN in complete Freund adjuvant) were tested at the dilutions indicated.

[b] Values of less than 20% are shown as 0.

R23, back-selected from SINv23, appeared antigenically indistinguishable from parental SIN. Another, SINv23/50-R23, was evidently a pseudorevertant which regained reactivity with mAb no. 23 but did not concomitantly regain other epitopes (defined by mAbs no. 18, 30, and 51) that had been altered in the initial selection of SINv23. The latter revertant, having been selected from a two-step mutant, remained unreactive with mAbs (nos. 50 and 49) defining the other E2 site.

Despite multiple plaque picks designed to ensure homogeneity of each variant virus population, it remained possible that parental SIN had been carried along in the SINv23 seed stock and that this, rather than a genuine revertant, had been recovered in the above process. The authenticity of SINv23-R23, in particular, might have been subject to question because it appeared antigenically indistinguishable from parental SIN. Fortunately, we were able to verify reversion by exploiting a prior observation. Early in this study we prepared two-dimensional oligonucleotide maps of the 26S mRNA of parental SIN and of selected variants. Relative to SIN (Fig. 2A), only one of the variants, SINv23, had a clear oligonucleotide shift (Fig. 2B) which could be highlighted by coelectrophoresis of parental and variant RNA (Fig. 2C). The large SIN oligonucleotide in question was isolated and partially sequenced. Instead of representing a potential coding region for an E2 neutralization site, the shifted oligonucleotide from SIN consisted of 5′-GACUA AUACUACAACAC . . .; this RNase T_1 fragment was localized in the 26S RNA, starting 24 nucleotides toward the 5′ side of the translation initiation site for capsid protein (14). Thus, we had identified a genetic marker which appeared to be unique to SINv23 but which was not implicated in the antigenic change. When SINv23-R23 was later analyzed, its RNA fingerprint (Fig. 2D) carried the fortuitous marker of SINv23, demonstrating that the revertant did not represent the prototype virus.

In addition to the marker oligonucleotide, another that varied in intensity among different fingerprints was absent in SINv23-R23 (Fig. 2D). Its sequence and therefore its significance are unknown. In any event, this oligonucleotide in itself could not have revealed unambiguous information about relationships between genotypic and phenotypic changes and illustrates further that RNA fingerprint analyses often have limited usefulness except in well-defined circumstances such as those described above.

The other revertant shown in Table 1, SINv23/50-R23, retained the oligonucleotide marker peculiar to SINv23 (data not shown). Moreover, the order of its selection and its unique antigenic phenotype (see above) point to its origin as a spontaneous revertant in the SINv23/50 population.

DISCUSSION

Antigenic variants of SIN, prototype of the *Alphavirus* genus, arose spontaneously in vitro and could be selected with individual N-mAbs. Moreover, it was possible, by consecutive selection, to obtain multisite antigenic variants including one which was altered in all three neutralization sites described here. Whether analogous mutation and selection phenomena contribute to antigenic differences among natural alphavirus isolates has not been established; nonetheless, the capacities of viable SIN mutants to accommodate multiple antigenic changes, thus rendering monospecific N-Abs to the original virus ineffectual, appear to have general implications for the design and use of alphavirus vaccines.

Reaction patterns between SIN variants and a battery of N-mAbs indicated that SIN possesses at least five independently mutable N-Eps clustering in three antigenic sites, one E1 and two E2 (Table 1). That number may represent an underestimate, as suggested by SINv33/50/23 escaping neutralization by all N-mAbs in this study while being neutralized (albeit less effectively) by hyperimmune sera prepared against parental SIN. It therefore could be inferred that SIN possesses one or more additional N-Eps not defined by our relatively small panel of N-mAbs; preliminary results (not shown) indicate that an E2-specific N-mAb (no. R6) characterized by Olmsted et al. (13) reacts with most and possibly all variants shown in Table 1. Such complexity had been inapparent in competitive binding assays, in which E2 N-Eps appeared to be intimately linked topologically (18; unpublished data). Molecular interrelationships among SIN N-Eps should become more clear once appropriate sequences are obtained and antigenic changes can be attributed to specific amino acid changes.

Because virus variants by themselves may prove inadequate for establishing causal relationships between genotypic and antigenic changes, it is desirable and perhaps necessary to obtain phenotypic revertants and ultimately compare their genotypes to those of parental and variant viruses. Presumably, revertants having ancestral antigenic phenotypes can arise spontaneously in variant virus populations which themselves resulted from point mutations. However, their isolation poses a formidable problem if such revertants have no competitive advantage which favors their enrichment from the dominant (variant) population. One solution, the positive selection of revertants on the basis of their attachment by solid-phase N-mAb, was presented here; thus, SINv23-R23 and SINv23/50-R23 were selected from variants and resemble either parental virus or, in the latter case, a new antigenic phenotype (Table 1). Since the isolation of antigenic revertants in this manner was novel, it was important that we were able to verify their origin from antigenic variants, thereby excluding the trivial possibility that they were reisolates of parental virus.

SIN neutralization remains a relatively ill-defined phenomenon. From the method of revertant selection, it is

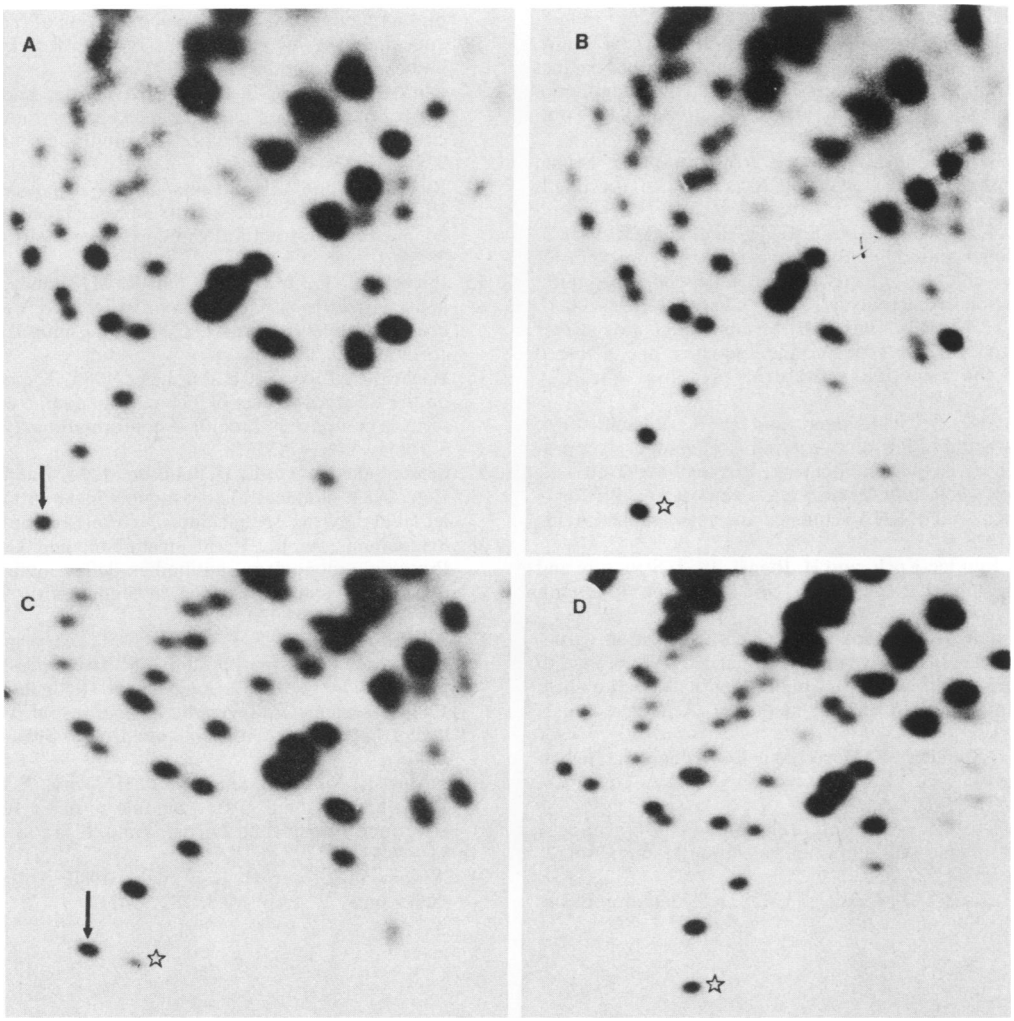

FIG. 2. RNA fingerprints distinguishing variant and revertant from parental SIN. Two-dimensional oligonucleotide maps represent the 26S RNA of SIN (A), antigenic variant SINv23 (B), coelectrophoresis of SIN and SINv23 (C), and the revertant SINv23-R23 (D). An incidental oligonucleotide marker confirmed that the back-selected revertant having prototype antigenicity arose from the antigenic variant and did not represent a reisolation of parental SIN. The marker SIN oligonucleotide (arrow), which was shifted (star) in the variant and revertant, presumably does not affect envelope antigenicity because it was localized just 5′ to RNA encoding the internal capsid protein; the subgenomic 26S mRNA is known to produce SIN structural proteins in the order 5′-C-E2-E1-3′ (8, 14).

evident that SIN was not rendered irreversibly noninfectious by attachment of E2-specific mAbs which, under different circumstances, clearly neutralized the virus. A similar paradox arises in the observation that the same N-mAbs, in low concentration (<1 μg/ml), enhance replication of SIN in an otherwise resistant Fc receptor-bearing cell line (G. A. Cole and A. Waddell, Abstr. 6th Int. Cong. Virol., 1984).

Except for their obvious involvement in neutralization, no biological functions could be assigned easily to glycoprotein regions that comprise SIN N-Eps. Independent changes in at least five different SIN epitopes were compatible with virus infectivity, and a possible limit to the number of acceptable alterations in N-Eps was not reached (Table 1). Furthermore, representative variants tested (data not shown) for their abilities to hemagglutinate goose erythrocytes were found to be unchanged with respect to activity (approximately 0.5 hemagglutination unit per ng of virus) or pH optimum (5.9), even though each of the N-mAbs described herein has hemagglutination-inhibiting activity which parallels its neutralizing activity (18; data not shown). Although it remains possible that some antigenic changes coincide with or cause alterations in SIN biology, many epitopes implicated in neutralization and hemagglutination inhibition may only adjoin or influence but not actually comprise sites having critical functions. Broadly interpreted, the above results, together with evidence that alphaviruses may have diversified (11) from a common progenitor (1) suggest that alphavirus N-Eps have considerable propensities to change without concurrent loss of virus infectivity.

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