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Virulence of La Crosse Virus Is under Polygenic Control

ROBERT S. JANSSEN,[1,2]† NEAL NATHANSON,[2]* MICHAEL J. ENDRES,[2] AND FRANCISCO GONZALEZ-SCARANO[1,2]

Departments of Neurology[1] and Microbiology,[2] School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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To identify which RNA segments of the California serogroup bunyaviruses determine virulence, we prepared reassortant viruses by coinfecting BHK-21 cells with two wild-type parents, La Crosse/original and Tahyna/181-57 viruses, which differed about 30,000-fold in virulence. The progeny clones were screened by polyacrylamide gel electrophoresis to ascertain the phenotype of the M and S RNA segments, and RNA-RNA hybridization was used to determine the genotype of selected clones. Two or three clones of each of the six possible reassortant genotypes were characterized quantitatively for neuroinvasiveness by determining the PFU/50% lethal dose (LD_{50}) ratio after subcutaneous injection into suckling mice. The reassortants fell into two groups. (i) Six of seven reassortants with a La Crosse M RNA segment were as virulent as the parent La Crosse virus (about 1 PFU/LD_{50}); the one exception was strikingly different (about 1,000 PFU/LD_{50}) and probably represents a spontaneous mutant. (ii) The seven reassortants with a Tahyna M RNA segment were about 10-fold more virulent than the parent Tahyna virus (median 1,600 PFU/LD_{50} for reassortants and 16,000 PFU/LD_{50} for Tahyna virus). A comparative pathogenesis study in suckling mice of one reassortant virus and the parent Tahyna virus confirmed the greater neuroinvasiveness of the reassortant virus. From these data it was concluded that the M RNA segment was the major determinant of virulence, but that the other two gene segments could modulate the virulence of a nonneuroinvasive California serogroup virus.

Segmented RNA viruses have provided powerful models for the study of the molecular mechanisms of viral pathogenesis because of the ease with which genetic reassortants can be constructed. Genetic analysis of reoviruses (5–7) has determined that the three genes which encode the outer capsid proteins are key determinants of important biological properties, such as tropism for specific cell types in the central nervous system, outcome of infection, ability to infect the gastrointestinal tract, and viral persistence. Genetic studies of influenza viruses (2, 22, 30, 31) have shown that virulence is determined by at least five of the eight viral RNA segments and that these segments interact so that the effect of a specific allele depends on the genetic background. Reassortants of lymphocytic choriomeningitis virus show (24, 25) that each of the two RNA segments carries certain virulence determinants; the S RNA segment, which encodes the N and GP proteins, mediates endocrine disease, and the L RNA segment, which probably codes for a polymerase, mediates guinea pig lethality.

Bunyaviruses (1, 3; D. Kolakofsky and B. Mahy [ed.], *The Biology of Negative-Strand Viruses*, in press) have three negative-sense single-stranded RNA segments. The L segment probably encodes the L protein, presumed to be the virion polymerase; the M RNA segment encodes two envelope glycoproteins, G1 and G2, and a nonstructural protein, NSm; the S segment encodes the nucleocapsid (N) protein and at least one nonstructural protein, NSs. The large family of bunyaviridae is divided into five genera; it appears that reassortants can only be made within each genus and probably only within a serogroup (11). We selected the California serogroup of the *Bunyavirus* genus for genetic studies because it has been shown by Gentsch and co-workers (9, 11–13) that reassortment readily occurred within this group of about 12 distinct viruses. Furthermore, the California serogroup offered two other important advantages. First, the prototype member, La Crosse virus, produces encephalitis in humans, and mice are a good model for this disease. Second, there are sufficient differences between La Crosse virus and other members of the California serogroup in both antigenic composition (15) and protein structure (1) to allow the typing of reassortant viruses by either their RNA (genotype) or their proteins (phenotype). Rapid typing was important because we wished to avoid temperature-sensitive (*ts*) mutants, which had been used in earlier studies (9, 11–13), since these may contain non-*ts* mutations which influence virus virulence (26).

For genetic studies we selected two viruses of markedly different virulence. The virulent prototype was La Crosse/original (LAC) virus, which will kill suckling mice after the extraneural (subcutaneous or intraperitoneal) injection of a minimal dose. The avirulent prototype was Tahyna/181-57 (TAH) virus, which is much less virulent than most strains of TAH virus and will kill suckling mice after extraneural injection only when a large dose is used. Detailed studies (19) of the pathogenesis of these two viruses showed that LAC virus replicated well in skeletal muscle and produced marked plasma viremia, while TAH virus did not. However, both viruses multiplied rapidly in the brain after intracerebral injection; thus, TAH virus was not neuroinvasive but was neurovirulent.

In the present study, reassortant viruses were constructed and tested for neuroinvasiveness. The data indicated that virulence is under polygenic control, although the M RNA segment is the major determinant of virulence.

* Corresponding author.
† Present address: Centers for Disease Control, Atlanta, GA 30333.

MATERIALS AND METHODS

Animals. Outbred albino Swiss mice (CD-1; Charles River Breeding Laboratories, Portage, Michigan) were used in all experiments.

Viruses and cell cultures. The origin and preparation of the LAC and TAH viruses have been described (19). Working stocks of both viruses were prepared as tissue culture supernatants from BHK-21 cells infected with twice-plaque-purified virus clones. Growth curve experiments with parent and reassortant viruses were done on BHK-21 cells (14).

Preparation of reassortant viruses. Recombination experiments were slightly modified from a previous protocol (12). Confluent monolayers of 2×10^5 BHK-21 cells in 24-well plates (Falcon Plastics, Oxnard, Calif.) were inoculated simultaneously with varying multiplicities of infection (MOI) of the parental viruses in Eagle minimal essential medium (MEM) with 2% fetal calf serum (FCS). After 2 h at 33°C, the supernatants with desorbed virus were removed and replaced with MEM with 2% FCS, and the cultures were incubated for 16 to 24 h at 33°C. The supernatants were harvested and stored in 20% FCS at −80°C, and clones were subsequently harvested from a standard plaque assay (19). Individual clones were grown on BHK-21 cells to make virus pools.

Determination of phenotype of reassortant viruses with PAGE. Confluent monolayers of BHK-21 cells in 24-well plates were inoculated at an MOI of 1.0 in 0.1 ml of MEM with 2% FCS and held for 30 min at room temperature. Medium was added to a total volume of 0.5 ml, and the monolayers were incubated at 35°C for 16 h. The supernatants were removed, and 25 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) in 0.5 ml of methionine-free medium (Gibco Laboratories, Grand Island, N.Y.) was added to each well. Following incubation at 35°C for 1.5 h, the supernatants were removed and the cells were lysed with Laemmli sample buffer (20). Each sample was boiled for 1 min and then subjected to polyacrylamide gel electrophoresis (PAGE) on a 10% acrylamide gel; the gel was treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.) and autoradiographed (15).

Determination of phenotype of reassortant viruses by ELISA with monoclonal antibodies. The method for preparing monoclonal antibodies and conducting the enzyme-linked immunosorbent assay (ELISA) has been described (14).

Preparation of ^{32}P-labeled vRNA. Monolayers of 5×10^7 BHK-21 cells in tissue culture flasks (T175; Nunc, Roshilde, Denmark) were inoculated at an MOI of 0.01 in 2 ml of MEM with 2% FCS. After 1 h at room temperature, the inoculum was removed and replaced with 15 ml of phosphate-free MEM with 5% FCS (FCS had been dialyzed against 6 liters of 0.9% NaCl for 48 h). After 2 h at 35°C, 2 mCi of sodium [^{32}]P_i (New England Nuclear) was added to each flask. After further incubation for 44 to 48 h, the supernatants were harvested at the time of maximum cytopathic effect and clarified by centrifugation at 2,800 rpm and 4°C for 20 min. Virus was purified in a 20 to 70% sucrose gradient (14). The visible band was removed in a 1-ml fraction and diluted to 2.5 ml in 150 mM NaCl–10 mM Tris, pH 7.4. Sodium dodecyl sulfate (SDS) was added to 1%, and virion RNA (vRNA) was extracted twice with phenol-chloroform (1:1), once with chloroform-isoamyl alcohol (24:1), twice precipitated with ethanol, and stored at −20°C. Incorporation of label into viral RNA was approximately 0.015%, and the harvest from 10 T175 flasks yielded 2×10^6 to 5×10^6 cpm.

Concentration of virus. Supernatants from infected BHK-21 cultures (two T175 flasks) were clarified as described above, overlaid on a 20% sucrose cushion, and pelleted at 27,000 rpm for 1.5 h. The pellets were suspended in 2 ml of MEM with 2% FCS, stirred at 4°C for 4 h, and sonciated with a Braun-sonic 1510 sonicator at 50 W for 4 s. Plaque assays usually indicated that the virus had been concentrated 5- to 10-fold.

Preparation of vcRNA. A published method (32) was used for the preparation of virion complementary RNA (vcRNA). Monolayers of BHK-21 cells in 10-cm petri dishes (Falcon) were inoculated at an MOI of 50 (with concentrated virus when necessary) and incubated for 6 h at 37°C. The cultures were transferred to an ice bath and washed three times with ice-cold phosphate-buffered saline. The monolayers were then lysed with 1 ml of lysis buffer (0.2% Nonidet P-40 in 30 mM Tris hydrochloride, pH 7.5, 150 mm NaCl, and 1.5 mm $MgCl_2$); the resulting suspension was centrifuged for 4 min at 2,800 rpm to pellet the nuclei. The supernatant was added to an equal volume of extraction buffer (0.2% SDS, 1 mM EDTA, 400 mM NaCl, 7 M urea, 10 mM Tris hydrochloride, pH 7.9). The RNA was phenol extracted and ethanol precipitated as described above. The yield from one petri dish was dried in a Speedovac (Savant Instrument Co., Hicksville, N.Y.) and suspended in 50 μl of 1 mM EDTA, pH 7.0.

RNA-RNA hybridization. The procedure for RNA-RNA hybridization was modified from that of Hay (16, 17). ^{32}P-labeled vRNA was mixed with unlabeled vcRNA, denatured, renatured, treated with S1 nuclease, and analyzed on an agarose gel. The hybridization mix contained 90,000 cpm of labeled vRNA, 30 μl of vcRNA, and deionized water to 50 μl. Dimethyl sulfoxide was added to 90% (vol/vol), and the RNA was denatured at 45°C for 30 min. The mix was diluted to 63% dimethyl sulfoxide with 5 mM EDTA–100 mM NaCl–40 mM Tris hydrochloride, pH 7.4, and held at 37°C for 14 to 16 h to permit the complementary RNA species to anneal. Following concentration by ethanol precipitation, the samples were suspended in 1 mM $ZnSO_4$–10 mM sodium acetate (pH 4.5) and digested with 1,000 U of S1 nuclease (PL Biochemicals, Milwaukee, Wis.) per ml for 6 h at 37°C. The samples were extracted with phenol, ethanol precipitated, dried, and suspended in MOPS buffer (50% formamide, 2.2 M formaldehyde, and MOPS [morpholinopropanesulfonic acid] buffer) at pH 7.0.

Agarose gel electrophoresis. RNA samples were subjected to electrophoresis on a 1% agarose–5% Formalin gel in MOPS buffer at 40 V for 16 h (21). The gel was dried, and autoradiographs were exposed for 6 to 8 h to visualize S RNA segments and for 16 to 24 h to visualize L and M RNA segments.

Virulence of reassortant viruses. Calibrated inocula of 0.5 $\log_{10}$ dilutions of selected viruses were injected subcutaneously in 72-h-old suckling CD-1 mice (two litters per dilution), and mortality was recorded daily for 9 days. A simultaneous plaque titration of the same virus dilutions was made, and the PFU-to-50% lethal dose (LD_{50}) ratio was computed. Probit analysis (8) was used to compute the PFU/LD_{50} ratios and 95% fiduciary limits. A logistic regression model for categorical data (18) was also fitted and provided a chi-square test for comparing groups of reassortants. Computations for these analyses were performed with the Statistical Analysis System (27).

RESULTS

Production of reassortant viruses and determination of phenotype by SDS-PAGE. Nonmutagenized clones of LAC

and TAH viruses were used to coinfect BHK-21 cells to produce reassortant viruses. The progeny clones from coinfection experiments were partially phenotyped by SDS-PAGE of ^{35}S-labeled viral proteins. Some of the gene products of the M segment (G1 protein) and the S segment (N protein) of LAC and TAH viruses migrate slightly differently, but their L proteins cannot be distinguished (1, 4, 10). The LAC virus G1 protein (120 kilodaltons [kDa]) migrates more rapidly than the TAH G1 protein (125 kDa), and the LAC virus N protein (24 kDa) migrates more slowly than the TAH virus N protein (21 kDa) (Fig. 1).

With this simple screening test 264 clones were partially phenotyped (the phenotypes and genotypes are expressed as a three-letter code, referring to the parental origin of the large [L], middle [M], and small [S] RNA segments, respectively, with L for LAC, T for TAH, and X for unknown) as follows: XLL, 55 clones; XTT, 172 clones; XTL, 5 clones; and XLT, 32 clones. Thus, at least 14% (37 of 264 clones) were reassortants, which was considerably lower than proportion expected (50%) assuming equal probability of all combinations. Reassortants of the phenotype XLT (31) predominated over reassortants of phenotype XTL (5); the latter were difficult to obtain even when the relative MOI of the two parent viruses was varied. The use of LAC virus-specific neutralizing monoclonal antibody did not increase the yield of reassortants of XTL phenotype (data not shown).

Viruses of phenotype XLL and XTT were a potential source of reassortants of genotypes TLL and LTT. However, this would have required determination of the genotype of a large number of viral clones by RNA-RNA hybridization. Since determining the phenotype was much easier, an alternative strategy was used. Two reassortants, one TLT and one TTL, were crossed; phenotyping the products of the M and S RNA segments was sufficient to yield a complete genotype. Among 52 clones, the distribution was TLT, 13 clones; TTL, 3 clones; TTT, 14 clones; and TLL, 4 clones. Likwise, a cross of two reassortants with genotypes LTL and LLT yielded 32 clones: LTL, 13 clones; LLT, 12 clones; LLL, 5 clones; and LTT, 2 clones. In summary, the parental genotypes predominated over the reassortant genotypes (58 to 25, although equal numbers of each were expected); among the four possible reassortant genotypes, the two desired genotypes were relatively infrequent (6 of 25 obtained, while 12 of 25 were expected).

Determination of genotype by RNA-RNA hybridization. To determine the genotype of reassortant viruses, we modified an RNA-RNA hybridization procedure originally described by Hay (16, 17). ^{32}P-labeled RNA was extracted from gradient-purified LAC and TAH virions and hybridized to unlabeled vcRNA obtained from lysates of infected BHK-21 cells. These hybridized RNA segments were subjected to single-strand-specific S1 nuclease digestion and analyzed on a denaturing agarose gel. Heterologous RNA molecules were degraded, while complementary RNA molecules were protected. Figure 2 demonstrates typical results for the parent LAC and TAH viruses and for several reassortants.

From the original crosses, genotypes of five XLT reassortants were determined, yielding three TLT and two LLT reassortants; all five XTL reassortants were genotyped, yielding three LTL and two TTL reassortants. Genotypes of seven XTT clones were determined, yielding one LTT reassortant. Further LTT and TLL reassortants were obtained by coinfection with reassortant viruses, as described above. At least two clones of each of the six possible genotypes were thus assembled (Table 1).

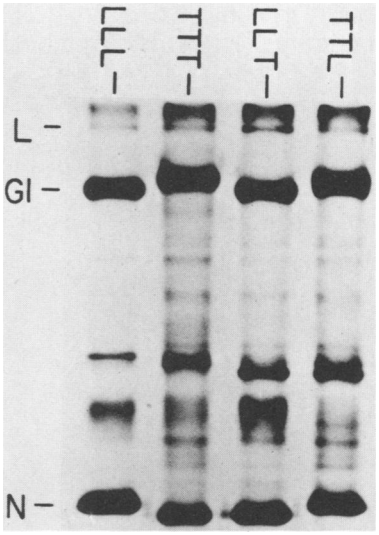

FIG. 1. PAGE of [^{35}S]methionine-labeled viral proteins from band-purified viruses. Viral proteins L, G1, and N are identified. (The G2 protein is not well defined in 10% acrylamide gels.) Genotypes of viruses are listed above each lane. Lanes 1 and 2 demonstrate the difference in migration of the G1 and N proteins of the parent viruses LAC and TAH. Lanes 3 and 4 demonstrate the migration of viral proteins from two reassortant viruses of known genotypes.

Growth in BHK-21 cells. To compare the relative ability of reassortant viruses to replicate, growth curves were compared for each parent and one clone of each of the six reassortant genotypes (Fig. 3). The parent LAC and TAH viruses grew at about the same rate for the first 36 h, but at 48 to 72 h LAC virus achieved titers about 10-fold higher than TAH virus. Reassortants with an LAC virus M RNA segment (LLT, TLT, and TLL) grew slightly less well than the LAC virus, and reassortants with a TAH virus M RNA segment (TTL, LTL, and LTT) grew slightly less well than the TAH virus.

Intracerebral virulence of reassortant viruses in suckling mice. To determine whether reassortant viruses maintained the high neurovirulence of both parental viruses, we titrated the parental viruses and six representative reassortant clones intracerebrally in weanling mice. The results are expressed as the PFU/LD_{50} ratio (Table 1). Log_{10} ratios ranged from about 0.3 to -0.6, and there were no statistically significant differences between the reassortants and the parental LAC and TAH viruses, all of which exhibited high neurovirulence.

Peripheral virulence of reassortant viruses in suckling mice. To quantitate the peripheral virulence of reassortant viruses, we titrated them by the subcutaneous route, using a protocol which minimized statistical variance. The log_{10} ratio of PFU/LD_{50} for the virulent LAC and avirulent TAH viruses was -0.3 and 4.2, respectively, a difference of about 30,000-fold. Reassortants with an LAC M RNA segment were very similar to the parent LAC virus. The single exception was clone P1-13c4, which was about 1,000-fold less virulent than the parent LAC virus. Reassortants with a TAH M RNA segment were about 10-fold more virulent than the parent TAH virus. The respective mean log_{10} PFU/LD_{50} ratios (and 95% confidence limits) for pooled data were 3.3 ± 0.13 and 4.2 ± 0.10; the difference was highly significant ($P < 0.001$).

Comparative pathogenesis of viruses of genotypes TTT and LTL. To confirm the observation that reassortant viruses

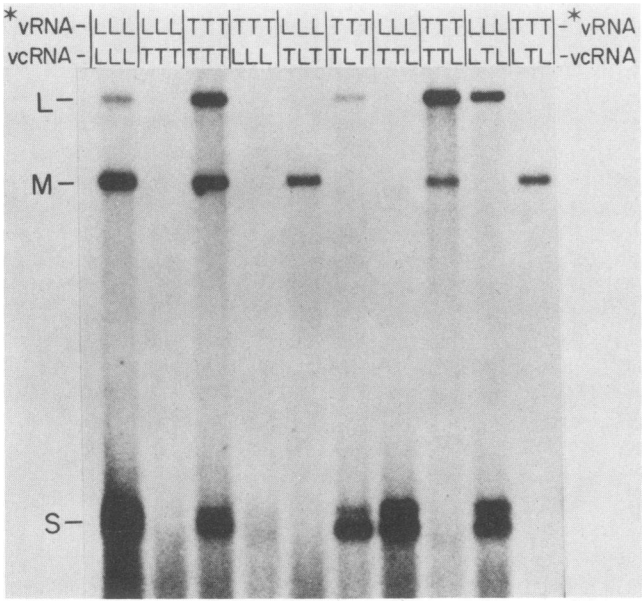

FIG. 2. Agarose gel electrophoresis of ^{32}P-labeled RNA segments from hybridization experiments to determine the genotype of reassortant viruses. Labeled vRNA from band-purified virus was hybridized to cold vcRNA from infected-cell lysates and treated with S1 nuclease. Following S1 digestion and phenol extraction, the samples were denatured and electrophoresed as single-stranded RNA segments. L, M, and S RNA segments are identified. Visible RNA segments on the autoradiogram represent homologous segments where the vcRNA protected the vRNA from S1 digestion. The S RNA segment ran as a double band because of differences in the size of two vcRNA species: antigenome was full length while mRNA was a subgenomic transcript. The genotype of the virus used to prepare both labeled (*) vRNA and unlabeled vcRNA is listed above each lane.

with a TAH M RNA segment were more virulent than the parent TAH virus, suckling mice were injected subcutaneously with 475,000 PFU of both the TAH virus and a reassortant virus of genotype LTL (F1-18a). The reassortant virus replicated somewhat more vigorously in peripheral tissues, with consistently higher virus titers in plasma and skeletal muscle (Fig. 4).

DISCUSSION

Construction of reassortant bunyaviruses and determination of their genotype. The present study extends the original investigations of Gentsch and co-workers (9, 11–13) on the genetics of reassortment within the California serogroup of viruses. The RNA segments and the structural proteins of the LAC and TAH viruses are quite compatible, since all six possible reassortant genotypes were obtained with relative ease and the reassortant viruses replicated in BHK-21 cell cultures almost as well as the parent viruses did. The overall frequency of reassortants was low relative to the frequency of progeny of the parental genotype. However, the frequency of reassortant progeny (at least 14%, versus 50% expected) was as high as that obtained in prior studies (9, 11–13). Our data demonstrate that it is practical to construct reassortant viruses without *ts* mutants, particularly when facilitated by the use of crosses between reassortant progeny. Pringle and co-workers, in crosses of bunyamwera group viruses (23), found linkage of the L and S RNA segments, while the M RNA segment of one virus reassorted freely with the L and S segments of another virus. Gentsch and co-workes (9, 12) had difficulty obtaining certain genotypes when they made reassortants between LAC and snowshoe hare viruses. Although the present data indicate that there are differences in the selection or replication of different reassortant viruses, there is no consistent pattern of genome segment linkage.

The construction of reassortant viruses from wild-type parents was markedly facilitated by a rapid method for typing progeny clones. The phenotypic differences in the protein products of two of the RNA segments make it possible to screen progeny clones by SDS-PAGE (23) and ELISA (15), but these methods do not distinguish the L proteins. Because of its simplicity and accuracy, we used an RNA-RNA hybridization method (16, 17) to determine the genotype of reassortant viruses. Gentsch and co-workers (9, 11–13) used RNA fingerprinting for the same purpose, but this technique is cumbersome for screening large numbers of progeny clones. Dot hybridization (23) of RNA with cDNA transcripts is an alternative attractive method. Pringle (23) observed that this method was not applicable to the S RNA segments of bunyamwera group viruses which have highly conserved nucleotide sequences, but we have found (unpublished observations) that dot hybridization will distinguish the S RNA segments of LAC and TAH viruses.

Genetic determinants of bunyavirus virulence. There were

TABLE 1. Subcutaneous virulence in suckling mice and intracerebral virulence in weanling mice of viral reassortants

Genotype	Clone	Source	Log_{10} PFU/LD_{50}	
			Subcutaneous (virulence)[a]	Intracerebral[b]
LLL	LAC	Parent	−0.3 (+)	0.0
TTT	TAH	Parent	4.1 (−) 4.2 4.3[c]	−0.6
TLT	B1-11a	LLL × TTT	−0.3 (+)	0.3
	B1-26a	LLL × TTT	0.2 (+)	
	B1-10a	LLL × TTT	0.3 (+)	
LLT	A1-3a	LLL × TTT	−0.3 (+)	0.3
	B1-29a	LLL × TTT	0.1 (+)	
TLL	P1-2gg	TLT × TTL	0.1 (+)	0.0
	P1-13c4	TLT × TTL	3.1[d] (+/−) 2.8	
LTL	F1-18a	LLL × TTT	3.2 (+/−)	−0.6
	F2-18a	LLL × TTT	3.2 (+/−)	
	F4-5a	LLL × TTT	3.1 (+/−)	
TTL	F1-2a	LLL × TTT	3.3 (+/−)	−0.5
	F2-2a	LLL × TTT	3.7 (−)	
LTT	B1-1a	LLL × TTT	3.9 (−)	0.3
	V1-1a	LTL × LLT	3.2 (+/−)	

[a] Virulence designation based on statistical test of differences between results for parent and reassortant viruses. Two coefficients of error for each determination were 0.25 log_{10} unit, and a difference of 0.5 log_{10} unit or greater was significant.

[b] Weanling mice were used for intracerebral titrations. Two coefficients of error for each determination were 0.5 log_{10} unit, and a difference of 1.0 log_{10} unit or greater was significant.

[c] Three separate determinations were made.

[d] P1-13c4 was significantly different from all other reassortants with an LAC M RNA segment. Two separate determinations were made. See text.

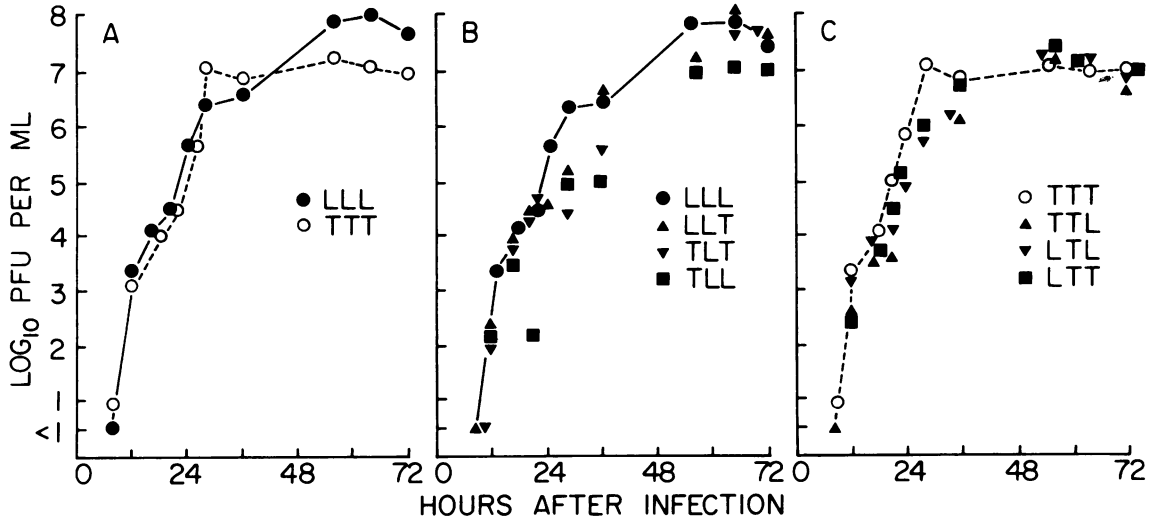

FIG. 3. Replication of LAC (LLL), TAH (TTT), and six reassortant viruses. Cultures were infected at an MOI of 0.01 and incubated at 35°C, and the culture medium was assayed for virus. (A) LAC and TAH (parent) viruses; (B) LAC and three reassortant viruses with an LAC M RNA segment (LLT, clone A1-3a; TLT, clone B1-11a; TLL, clone P1-2gg); (C) TAH and three reassortant viruses with a TAH M RNA segment (TTL, clone F1-2a; LTL, clone F1-18a; LTT, clone B1-1a).

two major findings in this study. (i) The M RNA segment is the major determinant of neuroinvasiveness within California serogroup bunyaviruses, in agreement with the prior findings of Shope and Bishop (23, 28–30). (ii) The L and S RNA segments modulate the effect of the M RNA segment in certain genetic backgrounds. This new finding is in keeping with genetic studies of other viruses (2, 5–7, 22, 24, 25, 30, 31), which demonstrate that virulence is usually under polygenic control.

There are several reasons for the differences between our findings and the earlier ones of Shope and Bishop (28). We maximized the difference in neuroinvasiveness between the two parental viruses by selection of a highly attenuated strain of TAH virus. The over 30,000-fold virulence difference between the two parental viruses allowed us to detect intermediate differences in neuroinvasiveness.

We constructed reassortants from wild-type parental viruses, minimizing the introduction of non-*ts* mutations into the genome of reassortant viruses; at least some of these *ts*-silent mutations can alter the biological properties of a virus (26). Shope and Bishop (28) could not compare their reassortants with the appropriate parental viruses since these parents were *ts* mutants; instead they were forced to compare reassortants with nonmutagenized wild-type viruses.

Our detailed pathogenesis studies (19) of LAC and TAH viruses showed that the ability of LAC virus to replicate in skeletal muscle and to produce viremia correlated with its neuroinvasiveness. Once within the central nervous system, TAH virus replicated slightly more rapidly than LAC virus (29), probably because the TAH strain was derived by multiple brain passages. It appears from the present data that the L and S RNA segments, as well as the M RNA segment, influence the rate of replication in muscle cells, but the

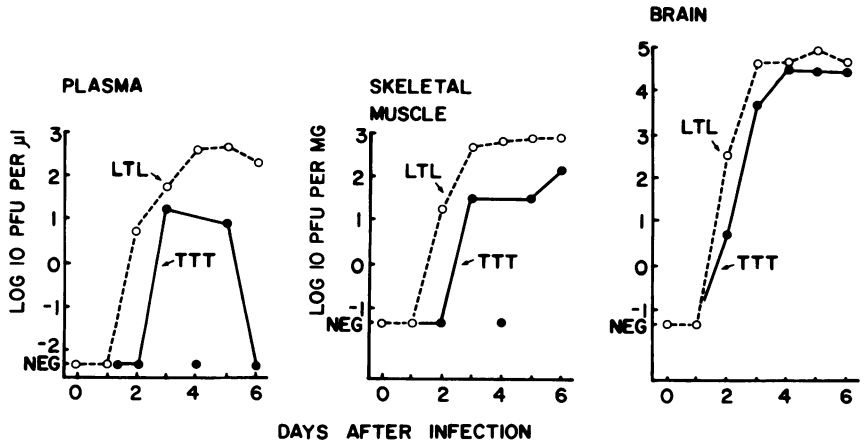

FIG. 4. Replication of viruses of genotype TTT (parent TAH) and LTL (clone F1-18a) in suckling mice after subcutaneous injection of 475,000 PFU. Titers are plotted per microliter of plasma or per milligram of tissue. Each point represents a pool of tissue or plasma for three mice. Sensitivity was 1 PFU/100 µl of plasma or 10 mg of tissue. Day 4 data for TAH virus (plasma and skeletal muscle) were not used in drawing the curves.

mechanism is not yet understood. Since virulent and avirulent viruses replicate in BHK-21 cells at almost the same rate, detailed investigation of the genetic basis of neuroinvasiveness will require use of myocyte or other cell culture systems which mimic the in vivo differences between viruses.

Certain limitations of the present studies should be recognized. We have been studying a single marker, neuroinvasiveness. The data already indicate that intracerebral virulence (neurovirulence) is a separate genetic marker which does not cosegregate with neuroinvasiveness. Genetic studies of a virus with low neurovirulence will be required to study the determinants of neurovirulence and its relationship, if any, to neuroinvasiveness. The importance of extending the present studies to additional viruses is suggested by the earlier observation of Shope and Bishop (29) that in reassortants between virulent LAC or snowshoe hare viruses and avirulent trivittatus virus avirulence was dominant, so that a reassortant virus with any trivittatus virus gene was avirulent. These preliminary observations stand in contrast to the present studies, in which virulence was dominant.

The availability of a panel of nonmutagenized reassortant viruses will also make it possible to address a number of questions about the biology of bunyaviruses; for example, which gene products are responsible for the turnoff of cellular protein synthesis; what is the role of the several small nonstructural proteins; what interaction is there between different RNA segments; and what are the determinants of tropism in vertebrate hosts? Such studies may indicate that a number of traits arise from the same gene products and require the construction of recombinant or mutant gene segments for further elucidation of the genetic control of biological properties.

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