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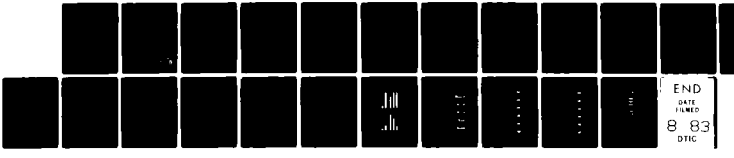

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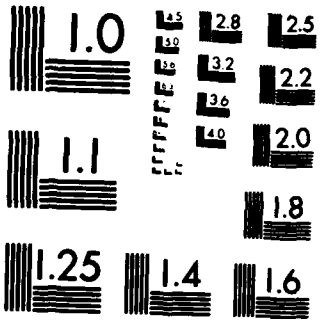

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In Vitro Selection of an Attenuated Variant of Sindbis Virus: Investigation of the Molecular Basis for Attenuation

Final Report

Robert E. Johnston

May 1982

Supported by

US ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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North Carolina State University
Raleigh, North Carolina 27650

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I. Introduction and Background

Adaptation of virulent viruses to an alternate host, either animal or tissue culture, has long been recognized as a means of attenuating virulence for the natural host. The molecular mechanism(s) involved in attenuation by blind passage in tissue culture is not understood. One explanation for this phenomenon is that virus replication involves many specific interactions between virus-derived and host-derived products. Selection in a particular host cell for virus mutants which have an increased efficiency in one or more of these virus-host interactions is thought to reduce the efficiency of the analogous interaction in another host cell type. Typically, selection of attenuated strains involves blind passage of virulent virus in a variable number of tissue culture cell lines. This is followed by cloning and testing for virulence in animal models. In general, many such passages are required to achieve attenuation, and no particular selective pressure is employed. Therefore, it has been difficult to examine the process of attenuation experimentally. We reasoned that if adaptation to specific host factors were involved in attenuation, then one could apply a selective pressure to enrich the population for virus mutants which exhibited the most efficient interaction with those factors. Efficient virus-cell interaction should lead to more rapid virus growth. Accordingly, we selected for those individuals in the SB population capable of the most rapid growth in BHK cells. We found that a stringent selection pressure for rapid growth led to early and significant attenuation of the virus.

We enriched for rapidly growing mutants by selecting for those progeny virions released from infected cells within 1-2 hours after the end of the latent period. These virions were used to initiate a second rapid-growth passage, and a rapidly-growing mutant was isolated by plaque-purification from the eleventh such passage (Figure 1). Compared to SB, this mutant (SB-RL) had a shorter latent period (hence SB-RL) in BHK cells (Figure 2A), and penetrated these cells much more rapidly than did SB (Figure 3). However, the SB and SB-RL latent periods and penetration kinetics were equivalent in a reptillian cell line (VSW), (Figures 2B and 3). SB-RL grew to higher maximal titers (Figure 2) and demonstrated higher rates of RNA synthesis than SB in all cell lines tested (Figure 4). The synthesis of both 42 and 26S RNA was increased (data not shown). SB-RL was not temperature-sensitive but was a small plaque mutant. SB-RL and SB had equivalent sedimentation velocities and bouyant densities; their RNA's appeared identical both in agarose-urea gels and by oligonucleotide T1 fingerprinting (D. W. Trent, personal communication), and their virion proteins were indistinguishable by SDS-polyacrylamide gel electrophoresis. However, we have demonstrated an antigenic difference between the two viruses using neutralizing antibody, suggesting that SB-RL and SB differed with respect to glycoprotein E2 (Table 1). An alteration in E2 could account for the accelerated penetration of SB-RL in BHK cells.

The virulence of SB-RL for neonatal mice was attenuated significantly compared to the virulence of SB. One day-old litters of ICR-L+ mice (1) were injected s.c. with doses of SB or SB-RL ranging from 2.5 to 2.5×10^6 PFU/mouse. In the case of SB, all animals died which received doses of 25 PFU or greater (Figure 5). The two mice which survived the lowest dose (2.5 PFU/mouse), had no neutralizing antibody to SB which suggested that they were not infected successfully. In this experiment, the survival rate of SB-RL infected litters was reduced from 60 to 30% over a dosage range of 6 logs. Therefore, mortality induced by SB-RL appeared to be only marginally dependent on dose, and it was not possible to obtain a classical LD_{50}. The mean day of death in SB-RL infected animals was extended significantly compared to mice inoculated with SB, 4.2±0.4 days for SB and 11.8±0.8 for SB-RL at a dose of 25 PFU/mouse. In addition, protection against SB challenge was established within 24 hrs.

following a primary inoculation of SB-RL (Figure 6). This suggested that SB-RL actively interfered with the replication of SB in vivo.

The forced adaptation of SB to BHK cells under a stringent selection pressure for rapid growth led to early and significant attenuation of the virus. We feel that the study of SB-RL replication in vitro and in vivo may offer a unique opportunity to study the process of attenuation of viruses in tissue culture and to identify a molecular marker related to virus pathogenesis. Sindbis virus is well characterized at the molecular level as well as in vivo, and the isolation of SB-RL has provided the additional tool required for this type of investigation. In addition, we feel that the study of this model system offers several practical advantages for the development of candidate live virus vaccine strains. First, the rapidity with which an attenuated strain was selected suggests that the procedure we have employed could significantly accelerate vaccine development during a military or public health emergency. Second, if attenuated strains selected in this fashion exhibit an effective in vivo interference as did SB-RL, then problems associated with reversion to virulence might be minimized. Finally, this same interference could provide protection during the period between vaccination and development of an active immune response.

II. Experimental Results

The objectives of this study have been 1) to determine the molecular characteristic(s) of SB-RL which is(are) responsible for its reduced virulence, 2) to examine the relationship between selection for rapid growth and selection for attenuation, 3) to define the pattern of SB-RL replication in vivo and 4) to determine the basis for protection of SB-RL inoculated animals against SB challenge. In this report, our progress toward these objectives will be discussed. However, it should be noted that the project was funded initially July 1, 1980, and this report covers only the one year period 7/1/80 through 6/30/81.

IIA. Pathogenesis of SB-RL in neonatal mice

Infection of neonatal mice with 1 PFU or more of Sindbis virus by either i.c. or s.c. routes led invariably to acute encephalomyelitis and death (4,3). The virus was isolated from virtually every organ with the highest titers found in the blood and brain (2). Histological examination revealed little evidence of pathological alterations in tissues of newborn mice infected by either s.c. or i.c. routes (2). Immunofluorescence studies of newborn mice injected s.c. showed that SB replicated initially at the site of inoculation followed within 24 hrs by widespread involvement of muscle, fibroblasts and endothelial cells of the small blood vessels (2,3). Spread of the infection to the central nervous system occurred via involvement of endothelial cells of the small cerebral vessels followed by rapid dissemination within the brain (2).

We have obtained similar results upon s.c. inoculation of SB (10^3PFU/mouse) into 2-day old ICR-L+ mice. Virus was detectable in the brain within 24 hrs post-infection and reached maximal levels at 3-4 days (Figure 7). Mean survival time was about 4 days and mortality was 100%. Histological examination of hematoxylin and eosin stained sections of brain tissue showed little or no pathological evidence of infection. However, virus antigen was detected by indirect fluorescent antibody staining in focal areas of infected brains 24 hrs after s.c. inoculation. By 2 days post-inoculation, much larger areas had become involved, and this generalized staining pattern was evident on days 3 and 4, as well.

SB-RL infection, however, presented a substantially different clinical picture.

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No SB-RL antigen was detected in the brain by indirect fluorescent antibody staining until 3 days post-inoculation, the areas of staining remained localized and no antigen was detectable after day 11. In contrast to SB infection which invariably resulted in death, SB-RL infection appeared to have three possible outcomes: one, a subclinical infection with no reduction in the normal pattern of weight gain; two, a clinical infection in which the animals exhibited reduction in weight gains, and either transient or permanent hind leg paralysis and seizures; and three, clinical infection ending in death. Consistent with these three alternatives, brain titers in SB-RL infected animals varied widely among individual mice, ranging from $<10^5$ PFU/gm to $>10^8$ PFU/gm in different mice on the same day after infection (Figure 8). However, the brain titers in particular animals were somewhat predictable by their weight and clinical symptoms, and were correlated strongly with the presence or absence of viral antigen as detected by fluorescent antibody. It appeared then, that the course of disease induced by SB-RL inoculation via a peripheral route was dependent upon whether or not the virus entered the brain. This supposition was supported by the finding that when SB-RL was inoculated i.c., the mortality observed was equivalent to wild-type (100%) with a mean survival of 3-4 days (Figure 9). However, reduced neuroinvasiveness does not completely explain our data. If once inside the brain, SB-RL were able to replicate as well as wild-type, then the distribution of viral antigen in the brains of animals inoculated s.c. with SB-RL would have been generalized as was the case with SB. We observed only focal areas of involvement even though in some of these animals, the virus titers were equivalent to those in SB inoculated mice.

We observed previously that mice inoculated with SB-RL were protected completely from challenge (s.c.) with SB 24 hrs later. This result suggested that SB-RL actively interfered with SB replication and that this interference might explain the self-limiting nature of SB-RL infection. We have explored this question further by decreasing the interval between primary inoculation and challenge (Figure 10). Significant protection from SB challenge was evident within 3 hrs after SB-RL inoculation. By 6 hrs, both survival time and mortality of the SB challenged group were equivalent to control animals inoculated with SB-RL but challenged with PBS. Figure 11 shows the results of an analogous experiment in which SB-RL inoculated animals were challenged s.c. with 10 LD_{50} vesicular stomatitis virus (VSV). If the protection against SB challenge resulted from an homologous mechanism, i.e. homologous interference or production of defective interfering (DI) particles, then we should have observed no protective effect against challenge with VSV. However, such an effect was found by 12 hrs post-inoculation with SB-RL, suggesting that the protective effect elicited by SB-RL against either SB or VSV challenge was mediated by an heterologous mechanism such as interferon (IF). With respect to extended survival, we found that SB was also effective in protecting against VSV challenge (Figure 12). Therefore, increased induction of IF by SB-RL compared to SB probably cannot account for the reduced virulence of SB-RL. Alternatively, SB-RL and SB may be differentially sensitive to IF, and/or the alteration in the E2 glycoprotein of SB-RL could limit its ability to invade and replicate within the brain.

IIB. <u>In vitro</u> correlates of attenuation

One objective of this study has been the identification of a molecular marker for attenuation in the Sindbis virus-suckling mouse system. We have proposed to approach this objective by treating the <u>in vivo</u> property of attenuation as a genetic trait of the virus. Comparison of SB and SB-RL as to their molecular composition and replication <u>in vitro</u> has described a spectrum of differences between the two. One or more of these differences could represent the expression of the attenuation marker in tissue culture. To identify the particular difference responsible for attenuation,

strains of SB-RL which have reverted to wild-type virulence in suckling mice will be isolated and characterized in tissue culture at the molecular level. The molecular characteristics of SB-RL associated with attenuation also should have reverted to wild-type in these strains. As an additional approach, virus clones have been isolated from earlier passages in the rapid growth passage series which led to the isolation of SB-RL (Figure 1). These will be analyzed to determine which properties they share with SB-RL and which they share with SB. Simultaneously, the virulence of these strains will be determined in vivo. Of the molecular characteristics which distinguish SB-RL from SB, those which are not found in attenuated strains from earlier passages will have been demonstrated to be superfluous to attenuation. Likewise, distinguishing molecular characteristics of SB-RL which are found in virulent strains from the earlier passages also will have been shown irrelevant to attenuation in vivo. If enough strains can be analyzed in this fashion, the spectrum of potential markers for attenuation described by SB-RL will be narrowed considerably. Reversion analysis can be applied to appropriate attenuated strains from the earlier passages to confirm conclusions reached by this approach.

Thus far, we have analyzed 39 isolates from rapid growth passages 4-12 with respect to virulence in 2-day old mice and plaque size in BHK cells (Figure 13). These results should be regarded as preliminary since the experiments included only a small number of mice and as we have had only a limited supply of animals for experimentation while we established our mouse colony. However, these data have revealed several points. First, each of the isolates appeared to fall into one of three categories when tested for virulence upon s.c. inoculation of 25 PFU/mouse: virulent (100% mortality, 4-5 day mean survival), marginally attenuated (80-90% mortality, 7-9 day mean survival) or attenuated (10-30% mortality, 12-13 day survival). (The observation period for these experiments was 14 days, so this value was used for the survivors in the calculation of mean survival time). Of 30 additional isolates from our wild-type SB stock, all were virulent. We also passed SB with an 18 hr growth period, and 6 isolates from the 11th such passage fell into the marginally attenuated category; none were attenuated to the extent of SB-RL. Second, selection for rapid growth in vitro appeared to be the operative pressure for the selection of attenuated virus. Attenuated clones were detected as early as passage 4, and the proportion of attenuated clones increased with passage. Third, although the proportion of small plaque clones also increased with passage, several clones were both small plaque and virulent. Therefore, neither small plaque size nor temperature sensitivity was correlated with attenuation in the case of SB-RL.

III. Discussion

The data which we have accumulated over the past 12 months have presented a rather complex picture of both SB-RL pathogenesis and the molecular basis for it. The finding of marginally attenuated strains suggested that a combination of factors may contribute to the attenuated phenotype of SB-RL. Such a multifactorial explanation may be required to account for 1) the absence of SB-RL in brain tissue of some s.c. inoculated animals, 2) the delay in invasion of the brain in other individuals, and 3) the limited replication of SB-RL in the brain once it has entered. A simple inability of SB-RL to infect neuronal cells is not likely as i.c. inoculation of SB-RL resulted in high mortality. Clearly, more extensive in vivo and in vitro characterization of SB-RL and related strains is needed before a cogent understanding of attenuation in this system will be forthcoming. In the second year of this contract, we propose to emphasize three areas of research which hopefully will complete portions of the fragmentary picture we now have. These are 1) comparative pathogenesis of SB, SB-RL and strains which are marginally attenuated, examining both s.c. and i.c. inoculated animals, 2) the relative roles of interferon and the immune system in protection of SB-RL inoculated mice against SB challenge, both s.c. and i.c., and 3) the in vitro characterization of SB-RL, virulent revertants of SB-RL, and marginally attenuated strains, especially with respect to their penetration kinetics, RNA synthesis and alterations in envelope glycoproteins.

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Table 1

Neutralization Index for Antisera to SB and SB-RL

	SB	SB-RL
Anti SB	1:800[a]	1:1600
Anti SB-RL	<1:100	1:3200

[a]Highest dilution of antiserum which neutralized 50% of the added virus infectivity.

Figure Legends

Figure 1. Selection for Rapid Growth.

SB (strain AR 339 from the laboratory of H. R. Bose) was passed in BHK cells (ATCC CCL 10) using a 5.5 hour interval for virus growth. SB-RL was cloned from the passage 11 stock by plaque purification.

Figure 2. Replication of SB and SB-RL.

Cell monolayers were infected with 5-10 PFU/cell. After a 1 hour period for adsorption, the inocula were removed, MEM-10 was added, and samples were taken at the indicated times for assay of infectivity. A. Replication in BHK cells; B. Replication in VSW cells. ● SB-RL; ○ SB.

Figure 3. Penetration rates of SB and SB-RL.

Cell monolayers were infected with SB or SB-RL at ∿150 PFU/monolayer. At the designated times, antiserum prepared against SB or SB-RL was added to neutralize any remaining extracellular infectious virus. After 10 minutes at 37C, the antiserum was removed, and the monolayers were overlayed with MEM containing 1% agarose for development of plaques. The number of plaques obtained after a 60 minute adsorption and no antiserum treatment was taken as the 100% value. ● SB-RL in BHK; ○ SB in BHK; ■ SB-RL in VSW; □ SB in VSW.

Figure 4. Rates of Viral RNA Synthesis.

BHK (panel A) or VSW cells (panel B) were treated with 5μg/ml Actinomycin D 2 hours prior to infection with SB or SB-RL. At the indicated times, the infected cells were pulse-labeled for 30 minutes with [^{3}H]uridine at 5μCi/culture and incorporation of radiolabel into TCA precipitable material was determined. ● SB-RL infected; ○ SB infected.

Figure 5. Mortality of Neonatal Mice Inoculated s.c. with SB or SB-RL.

One day old mice were inoculated s.c. with 0.05 ml of virus suspended in PBS, using at least 10 mice at each virus dose. The animals were observed for 14 days, and the fraction surviving at that time was noted. In other experiments we have observed infected animals for longer periods and only rarely have any deaths occurred after 14 days. ● SB-RL; ○ SB.

Figure 6. Protection of SB-RL Infected Mice from SB Challenge.

Panel A: Four day old mice were injected s.c. with 10^3 PFU/mouse SB-RL● ; 25 PFU/mouse SB○ ; or 10^3 PFU SB-RL + 25 PFU SB/mouse◑ . Panel B: Mice inoculated with 10^3 PFU/mouse SB-RL on day 4 ○ (redrawn from Panel A); mice inoculated with 10^3 PFU/mouse SB-RL on day 4 and 25 PFU/mouse SB on day 5 ◑ ; mice inoculated with PBS on day 4 and 25 PFU/mouse SB on day 5 ○ .

Figure 7. SB Replication in Brain Tissue of Neonatal Mice

Two day old mice were inoculated s.c. with SB at 10^3 PFU/mouse. At daily intervals two mice were sacrificed, and the virus titer in their brains was determined. Each point represents a single animal.

Figure 8. SB-RL Replication in Brain Tissue of Neonatal Mice.
Two day old mice were inoculated s.c. with SB-RL at 10^3 PFU/mouse. At daily intervals two mice were sacrificed, and the virus titer in their brains was determined. Each point represents a single animal.

Figure 9. Mortality of Neonatal Mice Inoculated i.c. with SB or SB-RL.
Two day old mice were inoculated i.c. with either SB or SB-RL suspended in 0.05ml PBS at the doses indicated. Deaths occuring within 24 hours were attributed to injection trauma and were not included in the data for virus inoculated mice.

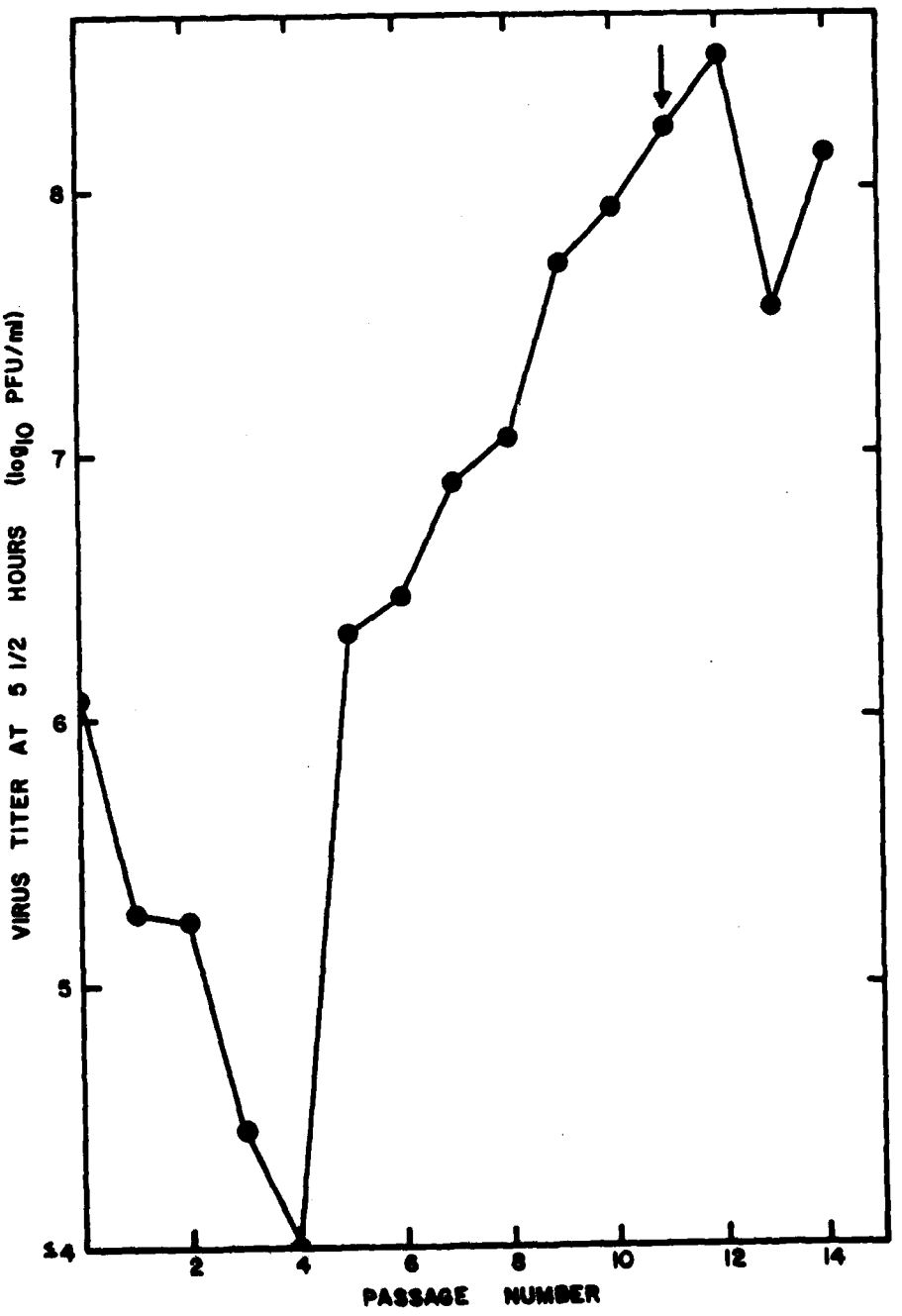
Figure 10. Protection of SB-RL Infected Mice from SB Challenge.
Two day old mice were inoculated s.c. with SB-RL at 10^3PFU/animal and then challenged s.c. with 25 PFU/animal of SB at the intervals indicated (shaded bars); mice inoculated with SB-RL and challenged with PBS (open bars); mice inoculated with PBS and challenged with SB (solid bars). At the 6, 18 and 24 hour challenge intervals, mortality in the challenged and unchallenged groups was equivalent.

Figure 11. Protection of SB-RL Infected Mice from VSV Challenge.
Two day old mice were inoculated s.c. with SB-RL at $5x10^2$ or $5x10^3$ PFU/animal and then challenged s.c. with 10 LD_{50}/animal of VSV at the intervals indicated. First bar (solid), PBS inoculation/VSV (10 LD_{50})challenge; second bar (upward shading), SB-RL ($5x10^2$)/VSV (10 LD_{50}); third bar (open), SB-RL ($5x10^3$)/VSV (10 LD_{50}); fourth bar (downward shading), SB-RL ($5x10^3$)/PBS. At the 18 and 24 hour intervals, in animals inoculated with $5x10^3$ PFU SB-RL and challenged with VSV, survival was 30 and 60%, respectively. Survival of animals inoculated with $5x10^3$ PFU SB-RL and challenged with PBS was 80 - 100% in this experiment.

Figure 12. Protection of SB Infected Mice from VSV Challenge.
Two day old mice were inoculated s.c. with SB at $5x10^2$ or $5x10^3$ PFU/animal and then challenged s.c. with 10 LD_{50}/animal of VSV at the intervals indicated. First bar (solid), PBS inoculation/VSV (10 LD_{50}) challenge; second bar (upward shading), SB ($5x10^2$)/VSV (10 LD_{50}); third bar (open), SB ($5x10^3$)/VSV (10 LD_{50}); fourth bar (downward shading), SB ($5x10^3$)/PBS.

Figure 13. Analysis of Other Isolates from the Rapid Growth Passage Series.
Several virus clones from passages 4 - 12 (Figure 1) were isolated by plaque purification, and stocks of these isolates were grown in BHK cells. Groups of two day old mice were inoculated s.c. with 25 PFU/animal. The observation period was 14 days, and this value was used for survivors in computing the mean survival time for each isolate. The darkened areas indicate the number of clones in each group, the injection of which allowed at least one survivor.

Figure 1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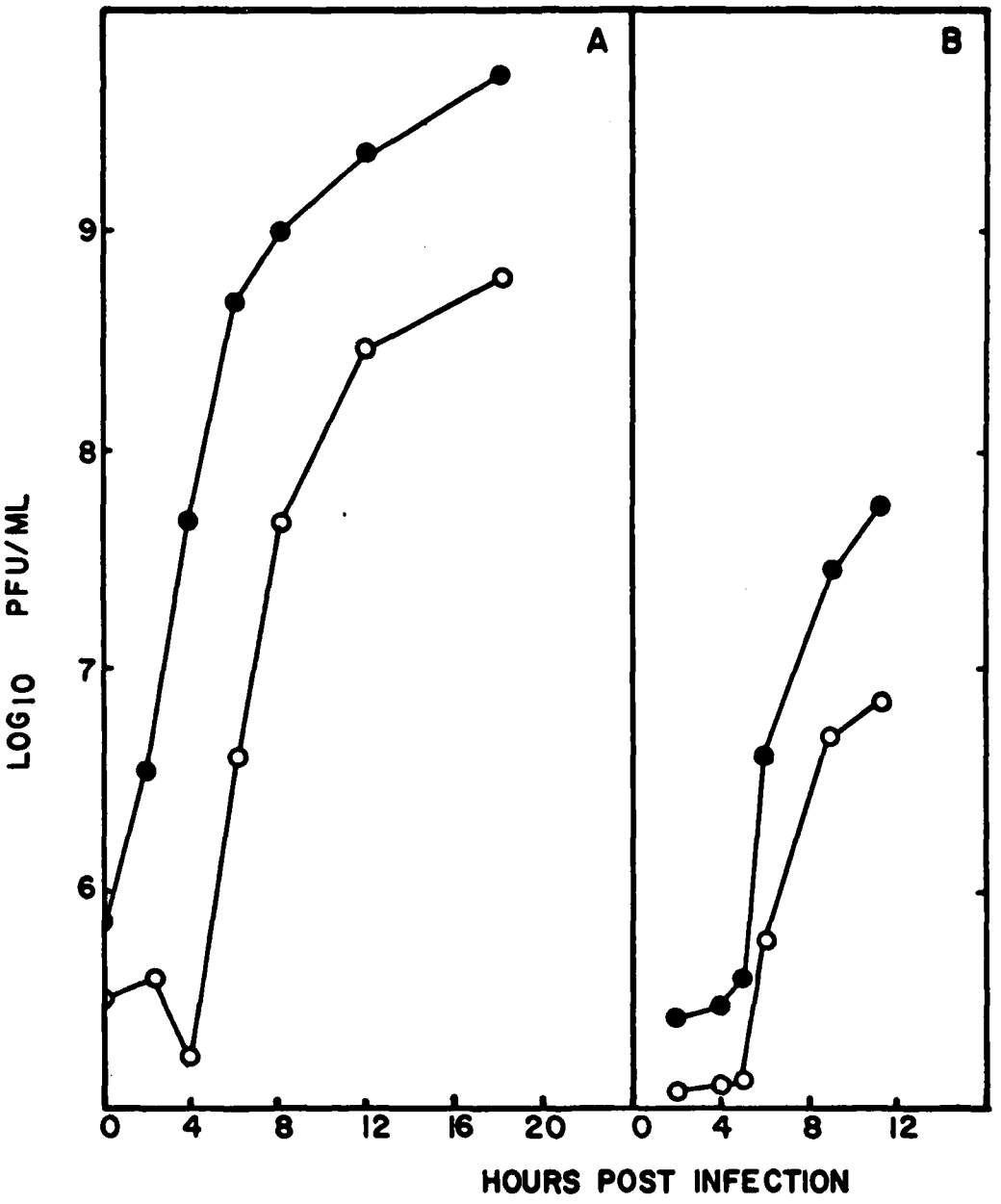

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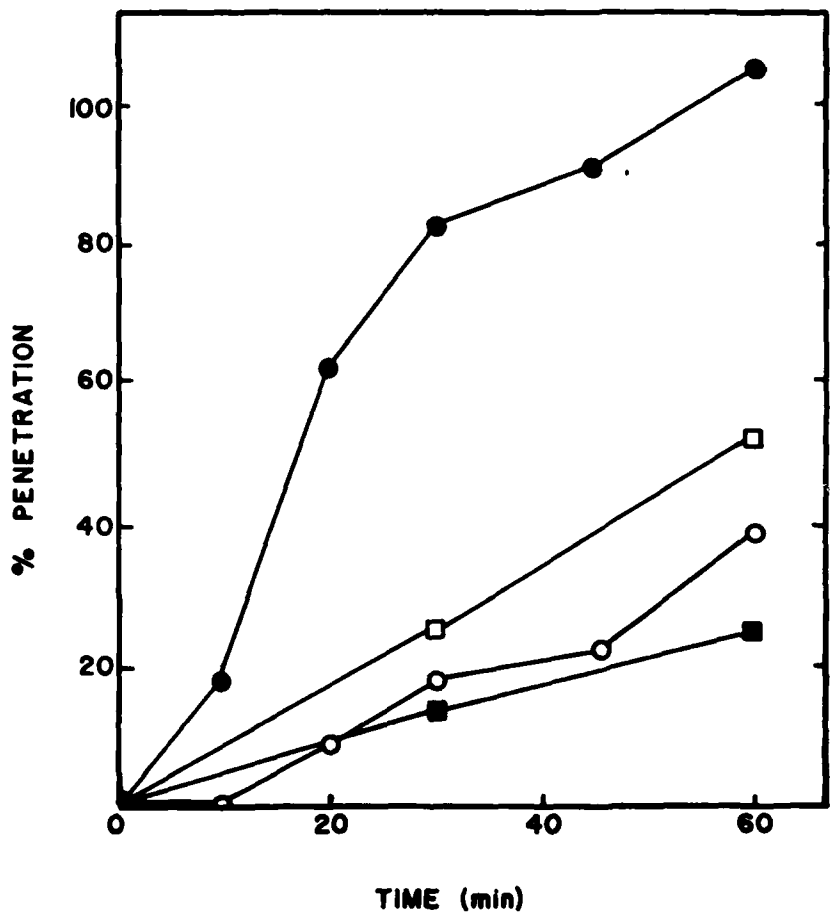

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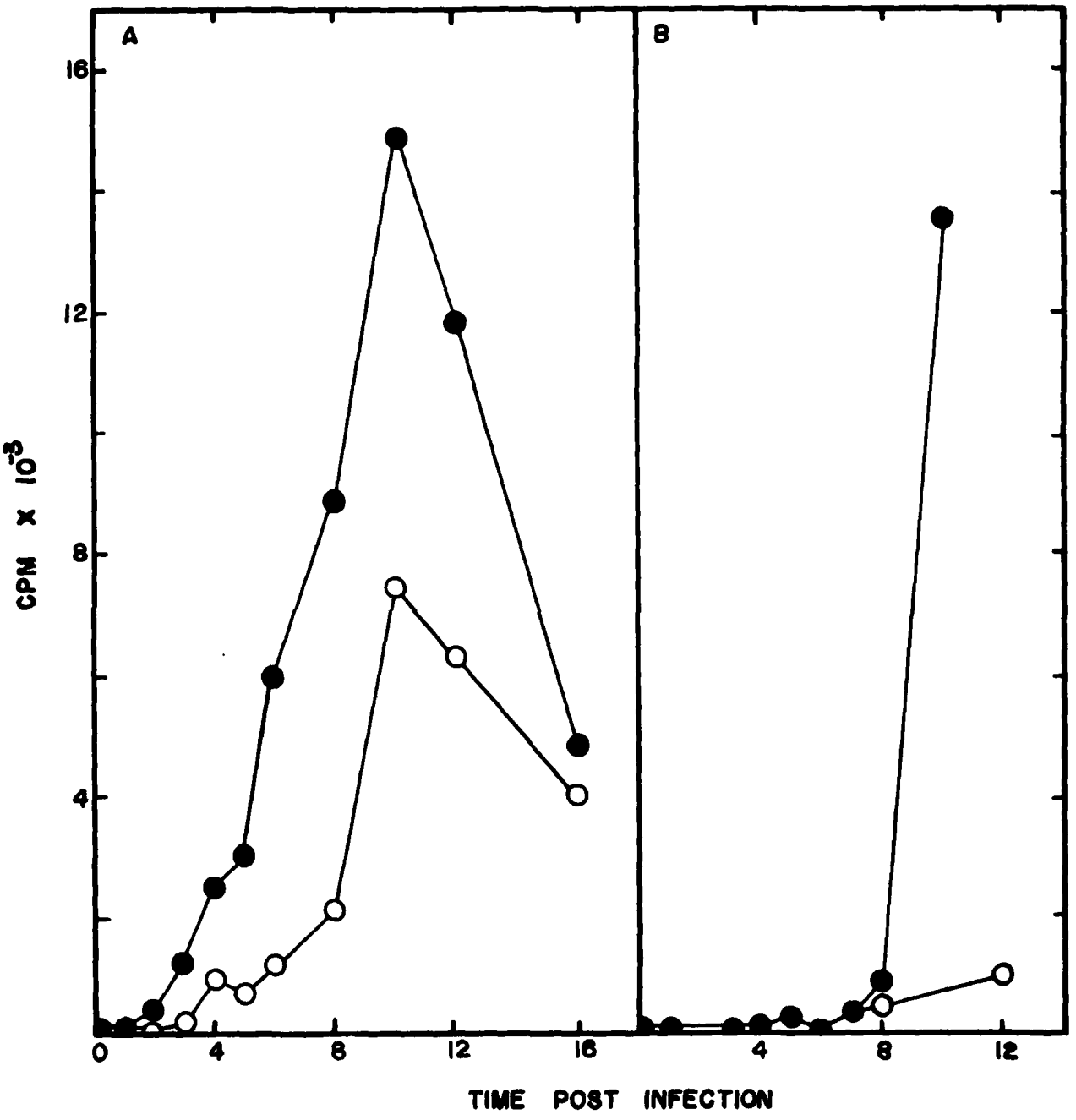

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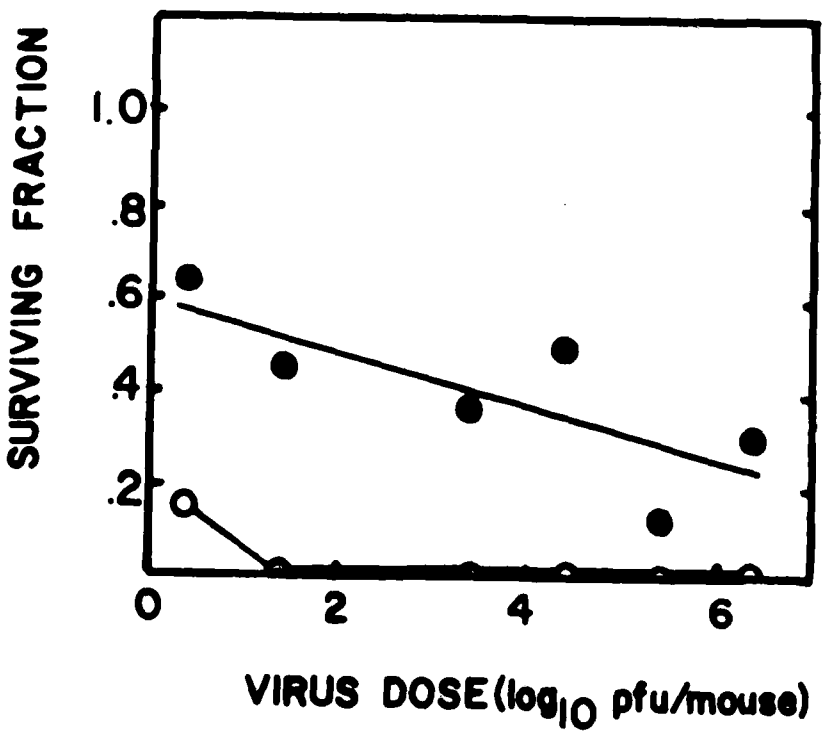

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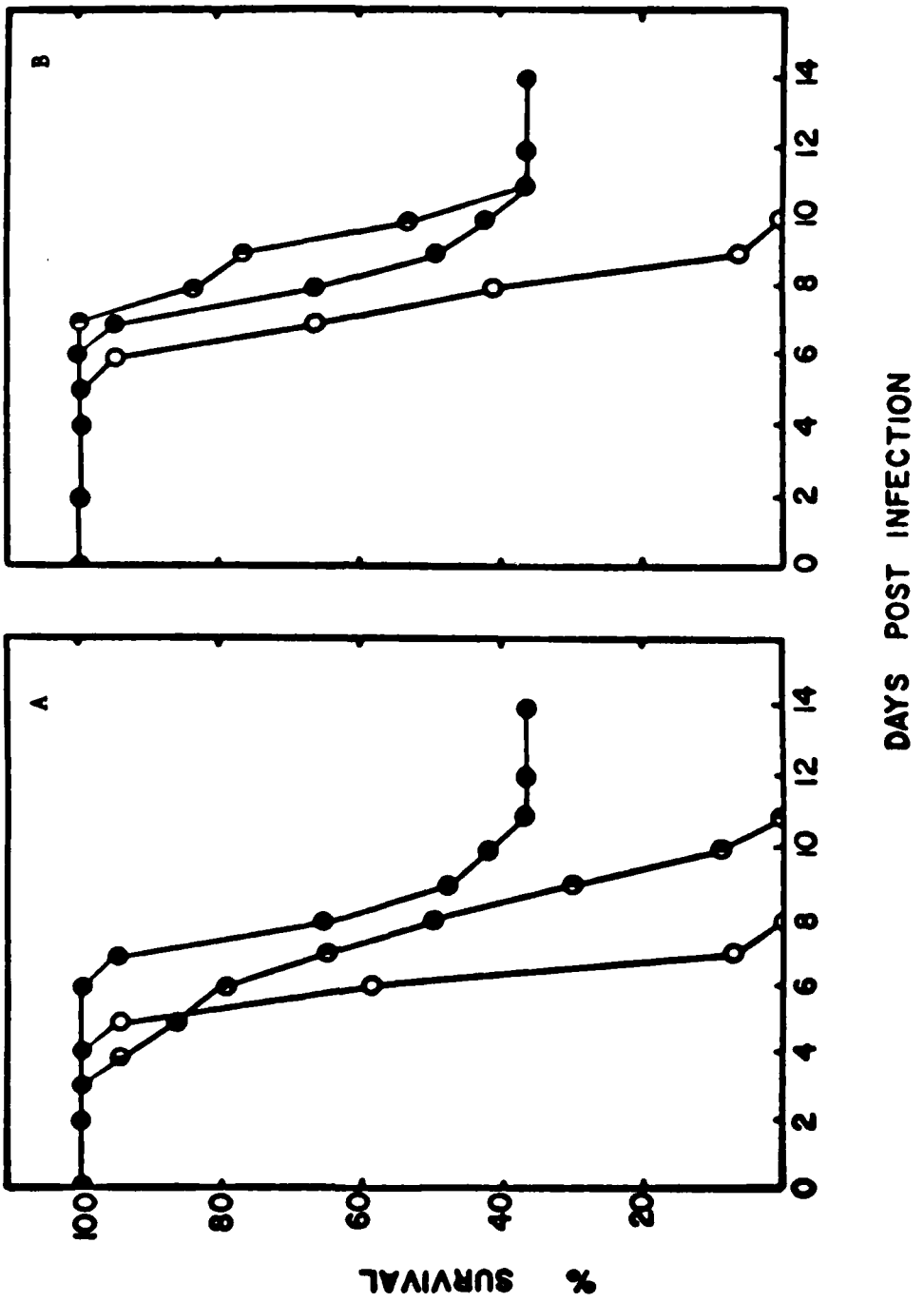

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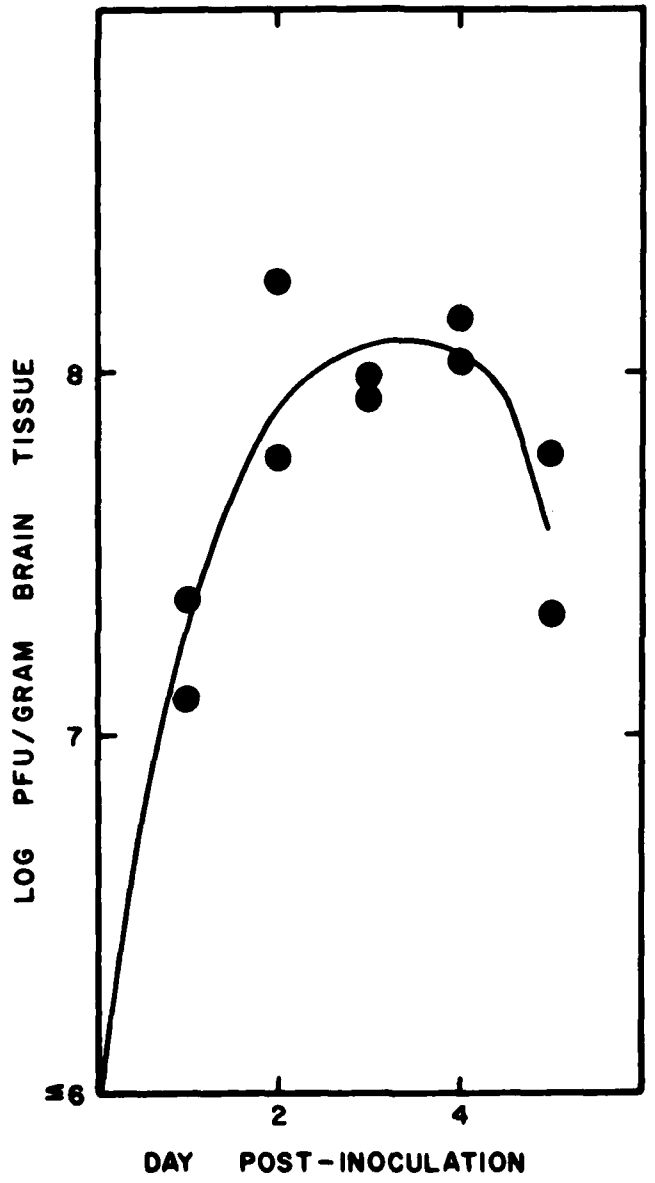

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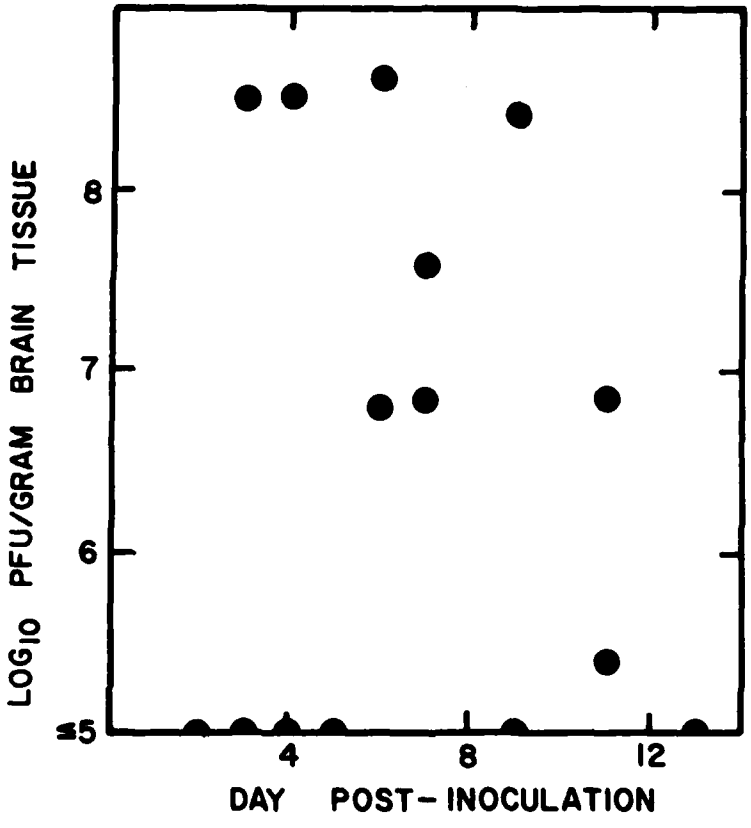

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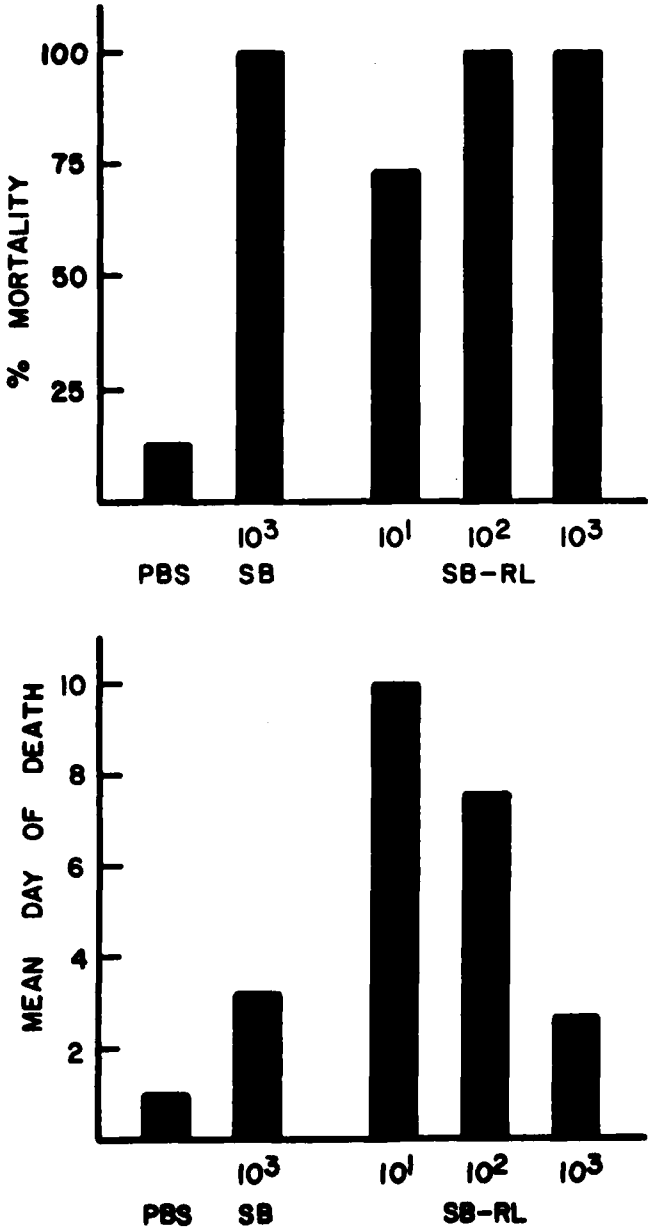
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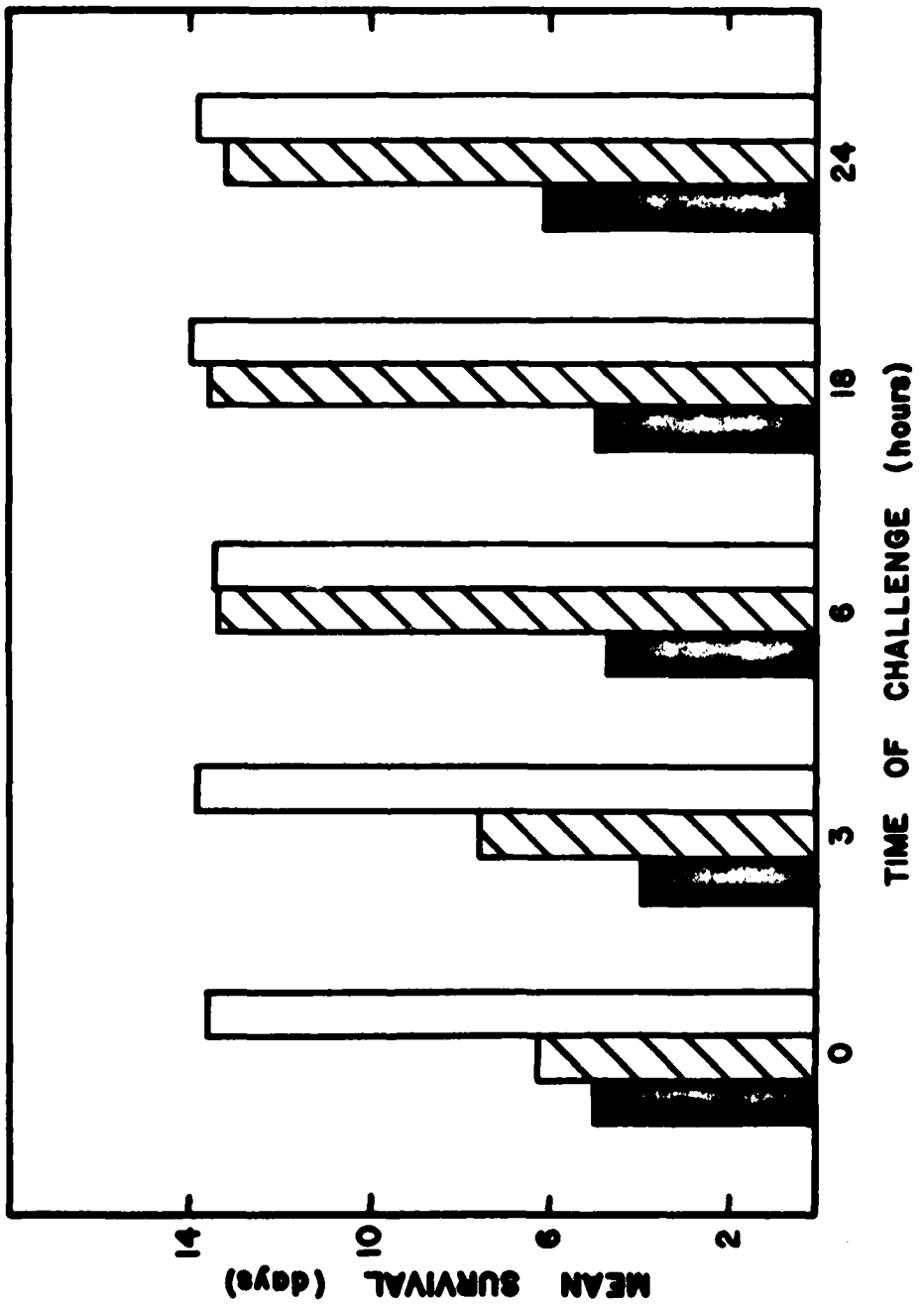
Figure 10



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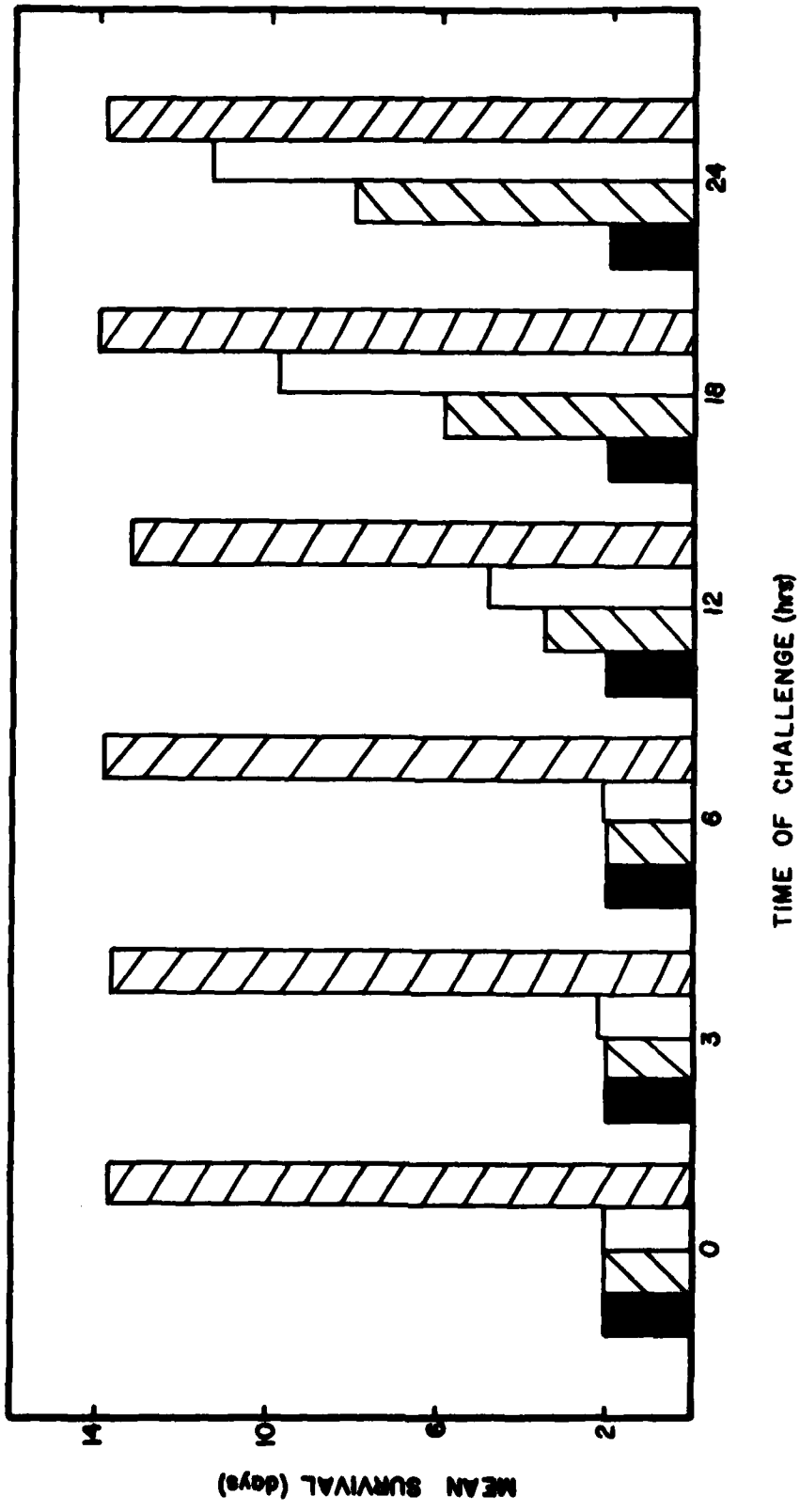

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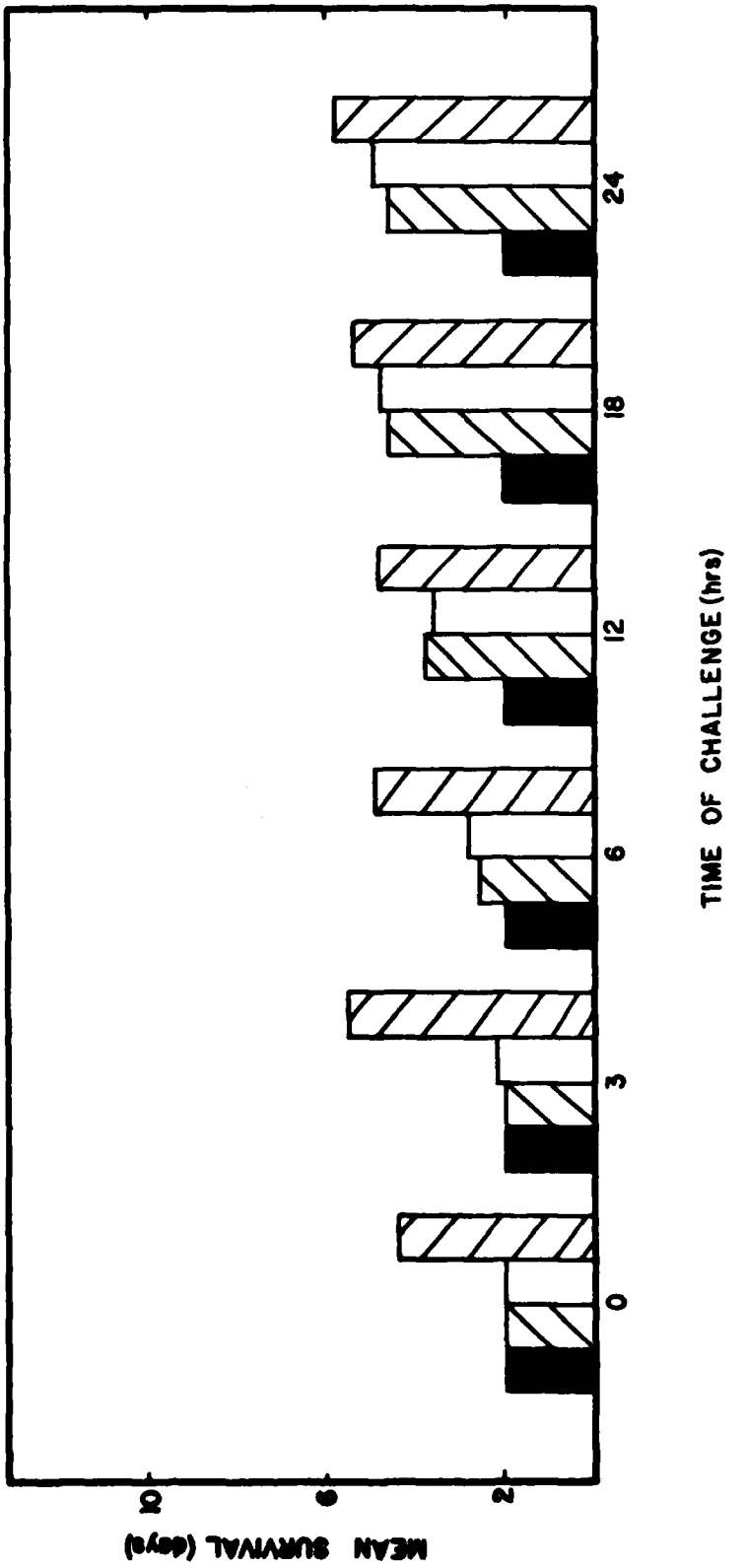
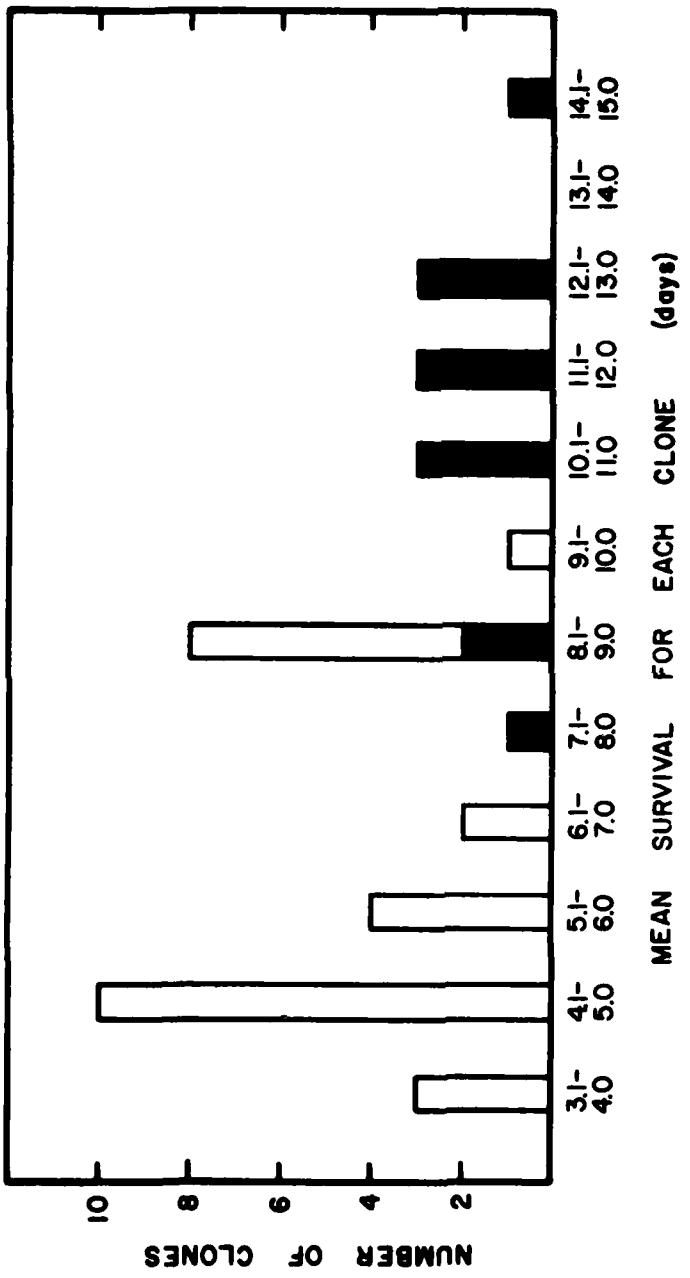


Figure 13


NUMBER OF CLONES
10
8
6
4
2
MEAN SURVIVAL FOR EACH CLONE (days)
3.1-4.0
4.1-5.0
5.1-6.0
6.1-7.0
7.1-8.0
8.1-9.0
9.1-10.0
10.1-11.0
11.1-12.0
12.1-13.0
13.1-14.0
14.1-15.0