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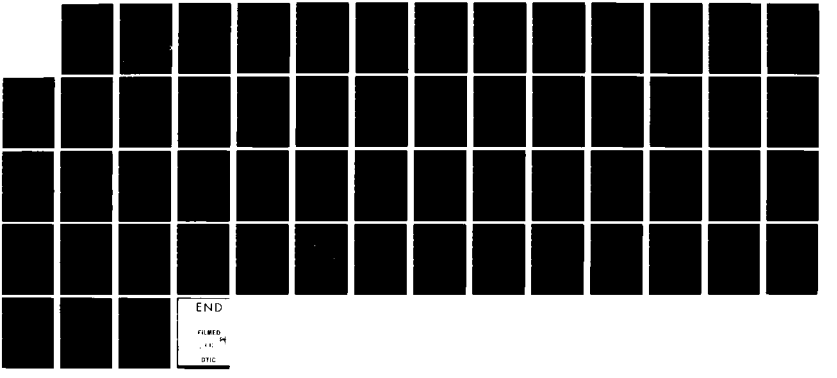

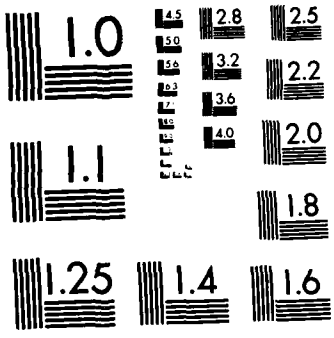

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Several mosquito cell proteins have been identified as being associated with (or incorporated in) Banzi virus grown in the polyclonal cell line of A. albopictus cells. This fact has been demonstrated by several observations. First, Banzi virions purified by polyethylene glycol precipitation and equilibrium density sucrose gradient centrifugation contain four mosquito cellular proteins. Second, anti-mosquito cell serum aggregates mosquito cell-grown Banzi virus, but not Banzi virus propagated in BHK cells. Third, anti-mosquito cell serum blocks the agglutination of erythrocytes by either unpurified or

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Report Number:

ROLE OF CELLULAR COMPONENTS OF MOSQUITO CELLS IN VIRAL REPLICATION AND TRANSMISSION

Annual Report

Final Report

Robert H. Schloemer

March 17, 1981

Supported by

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Indiana University School of Medicine
Indianapolis, Indiana 46223

Summary:

Several mosquito cell proteins have been identified as being associated with (or incorporated in) Banzi virus grown in the polyclonal cell line of A. albopictus cells. This fact has been demonstrated by several observations. First, Banzi virions purified by polyethylene glycol precipitation and equilibrium density sucrose gradient centrifugation contain four mosquito cell proteins. Second, anti-mosquito cell serum aggregates mosquito cell-grown Banzi virus, but not Banzi virus propagated in BHK cells. Third, anti-mosquito cell serum blocks the agglutination of erythrocytes by purified Banzi virus.

Uninfected mosquito cells secrete a complex of proteins which is able to agglutinate goose erythrocytes. The density of the complex and the pH optimum of its hemagglutinating activity are different than that of Banzi virus grown in mosquito cells for 24 hours. The same proteins (based on molecular weight determinations) which comprise the A. albopictus hemagglutinin are found in purified Banzi virus grown in mosquito cells.

Upon prolonged infection of mosquito cells with Banzi virus, the progeny virions differ in several respects. An apparent increase in the amount of one cellular protein is accompanied by a change in the pH optimum of agglutination of erythrocytes by Banzi virus. An increased reactivity of virus with anti-mosquito cell serum, in terms of neutralization of viral infectivity, is accompanied by a decrease in the ability of anti-Banzi viral serum to inhibit the hemagglutination activity of Banzi virus.

As a result of the incorporation of mosquito cell proteins into Banzi virions, mice immunized with uninfected mosquito cells or with purified A. albopictus hemagglutinin are protected against death due to Banzi viral infection. This protection is not due to any component of the medium and is specific for mosquito cell-grown Banzi virus. The protective effect afforded mice by prior immunization with mosquito cells is also observed after challenge with other togaviruses, but not with bunya- or rhabdo-viruses. Furthermore, immunization of mice with a monoclonal cell line of A. albopictus cells also protected mice against death due to Banzi viral infection.

Accompanying the changes in peptide profile of Banzi virus and in immunological reactivities of the virus is the appearance of soluble proteins in medium of mosquito cells infected with Banzi virus. These soluble proteins appear to be of viral origin, prevent Banzi virus production and decrease the number of mosquito cells releasing Banzi virus. This antiviral factor is specific for Banzi virus and has no effect on the replication of other togaviruses. This anti-viral factor has been purified and identified by tryptic peptides mapping and by immunological techniques to be very similar, if not identical to, V-1 protein found in purified Banzi virus.

The temporal relationship between (1) the change in the structure of Banzi virus during prolonged times of replication, (2) the production of a soluble viral protein capable of inhibiting Banzi viral replication and (3) the evolution of acutely infected mosquito cells into a population of persistently-infected mosquito cells suggests that the incorporation of host cell proteins into Banzi virus, maybe related to the establishment of a persistent state of viral infection in mosquito cells.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

FOREWARD:

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Faculties and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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PROGRESS REPORT

This report covers the period from March 1, 1977 to December 31, 1980.

A. Background and Statement of Problem

In nature, arboviruses are transmitted to vertebrates via arthropod vectors. Replication of flaviviruses in invertebrate hosts is poorly understood and differs remarkedly from growth of flaviviruses in mammalian cells. In particular, infection of mosquito cell lines with flaviviruses generally results in a low-titered, non-cytolytic, persistent infection which is in contrast to the destructive effects that these viruses have on vertebrate cell cultures. It is of interest that this response closely resembles the in vivo infections of mosquitoes in which arboviral infections are generally asymptomatic and life-long.

Replication of flaviviruses in A. albopictus cells differs from its replication in vertebrate cells in the following known ways. Progeny virus released from infected mosquito cells consists of only complete virus (RHA), a fact which is in contrast to the formation of both RHA and SHA in mammalian cells. In addition, progeny virions from infected mosquito cells are altered antigenically, probably as a result of host-cell mediated modification of the viral envelope, in that the hemagglutination (HA) activity of the A. albopictus cell-grown virus is blocked by antisera to uninfected A. albopictus cells. This result suggests that as flaviviruses mature and are released from mosquito cells, a host cell component becomes associated with the virus. A further observation which tends to support this hypothesis is that antiserum against A. aegypti mosquitoes neutralized infectivity of Sindbis virus which were propagated in mosquitoes.

The primary aim of this research project is to define the role of host cell components in the transmission and replication of viruses which are capable of growing in both arthropod and mammalian cells. Specific questions which were posed are as follows.

1) Do arboviruses incorporate host cell components into the virion as they replicate in mosquito cells?

2) If host cell components are incorporated into (or associated with) virions, is there any biological significance as related to transmission and replication of the virus?

3) What is the identity of the host cell components?

4) Is the incorporation of host cell components into virions related to the establishment of viral persistence to mosquito cells?

B. Results

1. Mosquito cell cultures. The mosquito cell cultures used in these experiments appear to be devoid of viral contaminants as determined by the following criteria: (1) supernatants from uninfected cells did not contain an agent which replicates in Vero or BHK cells as determined by plaque assays at 31 C or 37 C, (2) cocultivation of mosquito cells with either Vero, or BHK cells did not result in the formation of viruses which can be plaqued on or grown in A. albopictus, BHK or Vero cells, (3) injection of either culture medium of mosquito cells, or mosquito cells - intact or disrupted - into suckling mice did not result in death, (4) no syncytium formation in uninfected mosquito cells could be observed, (5) electron microscope observations of uninfected mosquito cells or of concentrated culture fluids failed to reveal the presence of any virus-like structures, (6) attempts to detect antigens of viruses used in this laboratory in uninfected mosquito cells by indirect immunofluorescence assays were unsuccessful.

Only the polyclonal A. albopictus cell line which was obtained from the American Type Culture Collection and the monoclonal cell line of A. albopictus cells (received from D.H.L. Bishop) were free of detectable contaminating viruses. In the subsequent experiments, the ATCC mosquito cell line was principally used. In those instances where the monoclonal cell line was employed, specific mention of this fact will be made.

2. Naturally occurring hemagglutinin of uninfected A. albopictus cells. On occasions, uninfected A. albopictus cells released a number of proteins into the medium; some of these proteins were capable of agglutinating goose erythrocytes at a pH optimum of 5.8. The production of this cellular hemagglutinin was variable (HA titers between <1:2 to 1:256), occurred in older monolayers, and was inhibited by actinomycin D. The complex of proteins agglutinating erythrocytes has a sedimentation coefficient of 20 to 25 S, and a density of 1.20 to 1.22 g/cc (see Figure 1C). Results of polyacrylamide gel electrophoresis of the cellular hemagglutinin purified by equilibrium density centrifugation revealed the presence of four proteins (see Figure 2C). These four proteins appear to be glycoproteins since [^{3}H] glucosamine radioactivity was present in each protein. Attempts to detect radiolabeled precursors to DNA and RNA in the purified A. albopictus hemagglutinin were unsuccessful. Thus, the cellular hemagglutinin appears to be a complex of four glycoproteins.

3. Polypeptide composition of Banzi virus grown in various cell lines. Banzi virus was grown in BHK-21 or in A. albopictus cells in the presence of [^{35}S] methionine. After 18-24 hours of infection, the extracellular virions were harvested and cellular debris was removed by centrifugation. Virions were concentrated by polyethylene glycol precipitation and subjected to equilibrium density gradient centrifugation for 40 hours. (This time of centrifugation was necessary to allow the complete separation of the cellular A. albopictus hemagglutinin from Banzi virions, and resulted in considerable loss of viral infectivity). Banzi virus which had been propagated in BHK cells (BV_{BHK}) uniformly banded at a density of 1.17 g/cc (Figure 1A). In contrast, Banzi

virions grown in A. albopictus cells ($BV_{A.albo}$) were distributed in a heterogeneous fashion and were present in two main regions of the gradient corresponding to densities of 1.22 g/cc and 1.17 g/cc (Figure 1B). Peaks of [^{35}S] radioactivity were coincident with peaks of infectivity (data not shown). The mosquito cell hemagglutinin isolated from uninfected cells exhibited a density of 1.21 g/cc to 1.22 g/cc (Figure 1C).

Virions present in regions of equilibrium density gradients which corresponded to a density of 1.17 g/cc were pooled, precipitated and subjected to electrophoresis on 8.5% polyacrylamide gels. BV_{BHK} contained three proteins designated V-3, V-2 and V-1 (Figure 2A), whereas $BV_{A.albo}$ contained four additional proteins, termed A-0, A-1, A-2 and A-3 (Figure 2B). It is of interest that the purified mosquito cell hemagglutinin (ρ=1.22) is composed of four proteins which appeared to have the same mobility in gels as those proteins associated with $BV_{A.albo}$ (Figure 2C).

The molecular weights of proteins found in purified Banzi virions grown in different cell lines and of proteins of the mosquito cellular hemagglutinin were estimated by comparing their electrophoretic mobilities to those of proteins of known molecular weights. A summary of the molecular weights is given in Table 1.

4. Banzi virus grown in mosquito cells contain cellular proteins. $BV_{A.albo}$ which had been labeled with [^{35}S] methionine was purified by equilibrium density centrifugation. Virions banding at a density of 1.17 g/cc were isolated, disrupted with detergents and incubated with either anti-Banzi viral serum or anti-mosquito cell serum. The resulting immune precipitates were collected, and analyzed by polyacrylamide gel electrophoresis. The results demonstrated that antiviral serum precipitated proteins designated as V-3, V-2, V-1 (Figure 3A). Identical results were obtained when BV_{BHK} were used (data not shown). Antiserum to mosquito cells precipitated proteins referred to as A-0, A-1, A-2 and A-3; no viral proteins were detected in the immune precipitates (Figure 3B). These results suggest Banzi virus which had replicated in A. albopictus cells contained host cell proteins.

To determine whether mosquito cellular proteins were associated with purified $BV_{A.albo}$, immune aggregation experiments were performed using [^{35}S] methionine labeled virus which had been purified by equilibrium density gradient centrifugation. Anti-Banzi viral serum precipitated intact virus grown in BHK-21 cells or in A. albopictus cells but did not aggregate the A. albopictus hemagglutinin (Table 2). Antiserum against mosquito cells precipitated native mosquito cell hemagglutinin and those intact Banzi virions grown in mosquito cells but did not react with BV_{BHK}. Results of polyacrylamide gel electrophoresis of aggregates which had formed after incubation of $BV_{A.albo}$ with either antiviral serum or anti-mosquito cell serum demonstrate the same viral and cellular proteins were found in aggregates formed by the addition of antiviral serum or of anti-mosquito cell serum to intact $BV_{A.albo}$ (data not shown). Polyacrylamide gels of aggregates formed after incubation either of BV_{BHK} with antiviral serum or of anti-mosquito cell serum with the

A. albopictus hemagglutinin are similar to Figure 2A and 2C respectively. Thus, these results provide further evidence that mosquito cell proteins are associated with Banzi virus.

The ability of the A. albopictus hemagglutinin to bind to Banzi virus was examined by immune aggregation experiments. We reasoned that if the presence of mosquito cell proteins with purified $BV_{A.albo}$ was due to the binding of the mosquito cell hemagglutinin to extracellular Banzi virus, then exogeneously added A. albopictus hemagglutin should bind to Banzi virus and should be precipitated by antiviral serum. Therefore, labeled mosquito cell hemagglutinin was incubated with purified, unlabeled $BV_{A.albo}$ or BV_{BHK} prior to the addition of antiviral serum. Alternatively, labeled BV_{BHK} were incubated with unlabeled, purified mosquito cell hemagglutinin prior to treatment with anti-mosquito cellular serum. The mixtures were centrifuged and precipitates were analyzed for radioactivity. As shown in Table 2, the addition of antiviral serum to those mixtures containing labeled mosquito cell hemagglutinin and unlabeled virus did not result in the precipitation of any radioactivity. These data suggest that exogeneously added mosquito cell hemagglutinin did not associate with Banzi virus grown wither in BHK cells or in A. albopictus cells. The fact that labeled BV_{BHK} preincubated with the mosquito cell hemagglutinin was not precipitated by anti-mosquito cellular serum also indicates the inability of the mosquito cell hemagglutinin to bind to the surface of Banzi virus grown in BHK-21 cells.

5. Apparent variation in polypeptide composition of Banzi virus grown in A. albopictus cells. Banzi virus was labeled with [^{35}S] methionine from 0-24 hours, 6-7 days or 59-60 days after infection of mosquito cells. The medium which contained extracellular virus was clarified by low-speed centrifugation, and then subjected to centrifugation at 125,000 x g for 4 hours. The pellets which contained the virions were resuspended in PBS and incubated with anti-Banzi viral serum. The resulting immune aggregates were collected and analyzed by polyacrylamide gel electrophoresis. The relative amounts of individual proteins in each viral preparation were normalized to the amount of V-2 present and are shown in Table 3.

The relative amounts of all proteins except for V-3 and A-1 were essentially the same in Banzi virus grown for 0-1 day, 6-7 day and 59-60 days in A. albopictus cells. A decrease in the amount of V-3 relative to V-2 was observed in virus which had been labeled after 6 days of infection. After 60 days of infection, V-3 could not be detected in Banzi virions. (It is important to note that this experiment using virus labeled from day 59-60 was done only once because of the huge amount of cells required to obtain sufficient virus.) However, it is evident that there appears to be an increase in the amount of A-1 associated with virus grown for 7 days as compared to the amount of A-1 associated with virus grown for 1 day. This increase in the relative amount of A-1 protein may reflect either an actual increase in the A-1 protein associated with the virus and/or a proteolytic cleavage product of V-3 which has the same molecular weight as the cellular protein (A-1).

6. Change in pH optimum of Banzi viral hemagglutinin. Purified Banzi virus grown in mosquito cells for 1 day has a pH optimum for hemagglutination at 6.5 (Figure 4A). In contrast, virus grown in mosquito cells for 7 days exhibited HA activity at pH 5.8 and 6.5; the highest iter was obtained at pH 5.8 (Figure 4B). The presence of HA activity at pH 5.8 is not due to free A. albopictus HA since the virus used in these experiments were isolated from equilibrium density at a density of 1.17 g/cc (see Figure 1C). These results may indicate that the A. albopictus hemagglutinin substitutes as the functional hemagglutinin on the virus. Virus which had been grown for 60 days in mosquito cells could not be obtained in sufficient quantities to perform these assays. However, purified Banzi virus grown in mosquito cells for 12 days was obtained in sufficient amounts to detect HA activity. As shown in Figure 4C, HA activity was detected only at pH 5.8. In a preliminary experiment, polyacrylamide gel electrophoresis of [^{35}S] methionine labeled virus grown for 12 days which had been purified by equilibrium density gradient showed reduced amounts of V-3 (data not shown). It should be noted that a rationale explanation for the reduced amounts of V-3 in virus grown for 12 days may be that the virus is unstable in sucrose and as a result is the purification scheme, a preferential loss (or removal) of V-3 may occur.

7. Effects of anti-mosquito cell serum on hemagglutination and infectivity of Banzi virus. Antiserum to uninfected A. albopictus cells was prepared in rabbits in the following manner. A. albopictus (2×10^8) were washed six times with warm PBS and disrupted by dounce homogenization. The nuclei were removed by low-speed centrifugation and the supernatant mixed with Freund's complete adjuvant was injected into the ear vein. After six weekly injections, serum was collected and complement was destroyed by heat treatment (56C for 30 minutes). The titer of this serum as determined by double diffusion in Outerlony plates was 1:64. The serum also contained antibodies to fetal calf serum; these antibodies were removed by adsorption to fetal calf serum. Experiments were conducted to determine whether this antiserum could inhibit viral hemagglutinating (HA) activity and viral infectivity.

Table 4 shows the effect of various antisera on the hemagglutinating ability of purified virus grown in BHK or A. albopictus cells. The observation that antiserum to A. albopictus cells inhibited the HA activity of Banzi virus grown in mosquito cells indicates that host cell proteins are associated Banzi virus grown in mosquito cells. Anti-mosquito cell serum had no effect on the HA activity of virus either grown in BHK cells or in mosquito cells and then passaged twice in BHK cells. Of interest, is that observation that anti-Banzi viral serum did not inhibit the HA activity of virus grown in mosquito cells but did block HA activity of virus grown in BHK cells. The reason for the lack of inhibition is unclear since Banzi virus grown for 24 hours in mosquito cells have V-3 which presumably is the viral hemagglutinin (see Figure 2B) and since antiviral serum does neutralize the infectivity of Banzi virus (see Table 5). These results only suggest that host cell proteins are associated with purified virus.

To demonstrate that the inhibition of HA activity of mosquito cell grown virus by anti-mosquito cell serum was due to the interaction of antibodies with host cell proteins on the virus, anti-mosquito cell serum was treated in a variety of ways prior to determining its ability to inhibit viral hemagglutination. The virus used in these experiments were purified Banzi virus grown in mosquito cells for 24 hours. Table 5 shows that the ability of the serum to inhibit viral HA activity is abolished upon pretreatment with either intact mosquito cells or with purified A. albopictus hemagglutinin, results which indicates that the inhibition of viral HA activity is due to the interaction of antibodies with cellular proteins on the virus. Furthermore, the fact that pretreatment of sera with trypsinized mosquito cell hemagglutinin did not abolish the ability of the sera to inhibit viral HA activity indicates that these antibodies react with a cellular protein (and not with glycolipids) on the virus.

The ability of anti-mosquito cell serum to neutralize the infectivity of Banzi virus grown in mosquito cells was investigated by plaque neutralization assays. Table 6 shows the effect of anti-mosquito cell serum on the infectivity of Banzi virus grown in various cell lines. In these assays, the end point was calculated as the highest dilution of serum which caused the neutralization of 50% of plaques. All sera were heat-inactivated (56C for 30 minutes) to destroy complement. The significant findings are 1) antiserum to mosquito cells neutralized the infectivity of only virus propagated in A. albopictus cells; it had no effect BV_{BHK} or on virus grown in mosquito and then passaged in BHK cells; 2) anti-BHK cellular serum did not neutralize the infectivity of virus grown in any cell line and 3) a difference in the ability of anti-mosquito cell serum to neutralize the infectivity of virus grown for 1 day or 7 days in mosquito cells is observed.

However, in plaque neutralization experiments using Dengue type II and Sindbis viruses grown in A. albopictus cells for 10 days, no significant neutralization of viral infectivity using anti-mosquito cell serum was obtained (neutralization titers of 1:20 were obtained).

Precubation of the antiserum with mosquito cells or with purified mosquito cell hemagglutinin resulted in the loss of the ability of the antiserum to neutralize viral infectivity (data not shown). These results indicate that antibodies present in anti-mosquito cell serum which neutralized viral infectivity are directed against mosquito cellular proteins.

8. Mouse protection experiments. Preliminary experiments have indicated that antibody response to A. albopictus cells in mice was obtained when mice were injected intraperitoneally with 10^7 washed, disrupted A. albopictus cells on days 0, 7 and 21. Sera which were collected from mice 7 days after the last injection was able to inhibit the hemagglutinating activity of Banzi virus grown in A. albopictus cells (HAI titer = 1024).

The ability of antibodies against A. albopictus cells to protect mice against infection by Banzi virus grown for seven days in A. albopictus cells was determined. Mice were immunized i.p. once a week for 5 weeks with either PBS (control) or A. albopictus cells which had been washed with PBS prior to disruption. One week later, mice were challenged i.p. with 1, 10 or 100 LD_{50} of Banzi virus. The results illustrated in Table 7 show the number of mice which survived 21 days after challenge. These results demonstrate that immunization of mice with uninfected mosquito cells does protect against infection with mosquito cell grown-Banzi virus.

Table 8 is a summary table illustrating the ability of various immunogens to protect mice from virus challenge employing 100 LD_{50} of Banzi virus grown seven days in A. albopictus cells or 24 hours in BHK cells. As noted previously, immunization of mice with A. albopictus cells results in protection against challenge by A. albopictus cell-grown virus, and not by BHK cell-grown virus. Prior immunization of mice with BHK cells does not afford protection against virus challenge. The observed protection against challenge with mosquito cell grown virus in mice which were previously immunized with mosquito cells is not a result of a component of the medium adhering to both virus and cell surfaces because of the lack of protection to virus challenge in mice immunized with media used to cultuvate A. albopictus cells. Of significant interest is the observation that mice immunized with purified A. albopictus hemagglutinin are protected against death caused by mosquito cell grown virus.

To determine whether immunization of mice with A. albopictus cells with protect mice against death by other viruses capable of replicating in mosquito cells, experiments similar to those described above were conducted employing Eastern equine encephalitis virus (alphavirus), Japanese encephalitis virus (flavivirus), Germiston virus (bunyavirus) and vesicular stomatitis virus (rhabdovirus). The results illustrated in Table 9 demonstrate that mice immunized with mosquito cells survived challenged with Eastern equine encephalitis and Japanese encephalitis viruses. All other permutations of (i) cell line used as the immunogen (ii) challenge virus and (iii) cell line employed for viral propagation resulted in death of mice.

To ascertain whether the observed protection against togaviral challenge is "unique" to the ATCC A. albopictus cell lines, similar protection experiments were performed with a monoclonal A. albopictus cell line. Preliminary experiments indicate that this monoclonal cell line produces a cellular hemagglutinin whose pH optimum is 5.8. As shown in Table 10, immunization of mice with the monoclonal cell line also resulted in protection of mice against death due to Banzi virus

9. Neutralization of Banzi virus grown in intact mosquitoes by anti-mosquito cell serum. In an initial experiment, whole mosquitoes (A. albopictus) were injected with Banzi virus. This was done by Dr. Robert Tesh of the Pacific Research Corporation. After 15 days of infection, mosquitoes were killed by placement in liquid nitrogen. Fifteen mosquitoes were ground in 2 ml of BME-Hank's balanced salt solution. After centrifugation to remove cellular debris, virus was titered on BHK-21 cells. Only 2.1×10^3 PFU were recovered. This amount allowed for one experiment in which aliquots of virus were treated with either anti-mosquito (A. albopictus) serum or with anti-Banzi virus serum. Banzi virus grown in whole A. albopictus mosquito were neutralized by both anti-A. albopictus cell serum and anti-Banzi virus serum. A 1:80 dilution of anti-mosquito cell serum caused a 55% reduction in the number of plaques. This experiment was not repeated since insufficient amounts of the virus remained.

10. Characterization of mosquito cells infected with Banzi virus. Since the polypeptide composition of Banzi virus appears to change as the virus replicates for long periods of time in mosquito cells, we investigated the possibility that this change is related, in some manner, to the development of viral persistence. Therefore, the growth of Banzi virus in mosquito cells was examined at regular intervals for a period of 91 days after infection. The amount of virus produced in mosquito cells was maximal (1×10^9 PFU/ml) at about 24 hours post-infection, and progressively declined until day 21 from which time the titer remained relatively constant at 5.5×10^4 PFU/ml. Similarly, the number of cells which released virus, as determined by infectious center assays, reached a maximum at 24 hours and declined until day 21 from which time, the number of cells releasing virus remained constant at about 1%.

To ascertain whether mosquito cells infected for 90 days were persistently infected with Banzi virus, the ability of these cells to support the replication of superinfecting togaviruses was examined (Table 11). Mosquito cells infected with Banzi virus for 90 days excluded the replication of superinfecting Banzi virus, but were as susceptible to infection by Eastern equine encephalitis virus or Japanese encephalitis virus as uninfected (fresh) mosquito cells. Since cells infected with Banzi virus for 90 days were resistant to superinfection by the homologous virus, produced less virus than acutely infected mosquito cells and less than 1% of these cells secreted virus, these cells were judged to be persistently infected with virus.

In other experiments, cells infected for 60 or 90 days with Banzi virus appeared to have the same gross morphology and generation time as uninfected mosquito cells (data not shown).

Another assay employed to determine whether cells infected for 60 or 90 days were persistently infected with Banzi virus was to determine the number of cells containing viral antigens as assayed by indirect immunofluorescence procedures. It was expected that approximately 1% of these cells should contain viral antigens since only 1% of cells produced virus. However, the same number (85-93%) of cells infected with Banzi virus for 60 or 90 days contain viral antigens as did acutely infected cells (24 hour postinfection). Uninfected mosquito cells did not react with antiviral serum. The fact that prior adsorption of antiviral serum with infected mosquito cells abolished subsequent fluorescence in acutely infected mosquito cells or in Banzi virus-persistently infected (BVPI) mosquito cells suggests that this assay is specific for Banzi viral antigens.

11. Soluble viral antigens in medium of mosquito cells persistently infected with Banzi virus. To account for the observation that less than 1% of BVPI mosquito cells secrete virus and approximately 90% of these cells contain viral antigens, we proposed that the 1% of cells producing virus also secrete a soluble viral protein into the medium. The viral protein would be able to bind to uninfected cells. To test this hypothesis, culture fluids of persistently infected mosquito cells (60 days of infection) and of uninfected mosquito cells were subjected to filtration through an Amicon XM100 filter.

The resulting filtrates which contained material of 100,000 daltons or less were incubated with uninfected mosquito cells for 2 hours. The binding of viral proteins in the filtrates of cells was detected by immunofluorescent procedure using antiviral serum. Cells treated with filtrates of medium of uninfected cells did not react with antiviral serum. However, 95% cells treated with filtrates of BVPI mosquito cells did react with antiviral serum. These results suggest that a viral protein(s) is being secreted by BVPI cells and binds to uninfected mosquito cells.

12. Ability of soluble viral proteins to inhibit Banzi viral replication. Culture media of uninfected mosquito cells and of mosquito cells infected with Banzi virus for either 24 hours (acute infection) or 90 days (persistent infection) were partitioned by dialysis into a fraction which contained molecules of 12,000 daltons or less. This fraction contained viral antigens which were able to bind to uninfected mosquito cells as determined by indirect immunofluorescence assays using anti-viral serum (data not shown). The fractions of various culture media were applied to uninfected mosquito cells for 24 hours prior to infection with Banzi virus. Virus yields and infectious centers were determined 24 hours postinfection. Table 12 shows that the medium of Banzi virus-persistently infected (BVPI) mosquito cells inhibited virus production by 99.6% whereas medium of either acutely infected or uninfected mosquito cells had no significant inhibitory effect on Banzi viral replication. In addition, medium of BVPI mosquito cells caused a 95% decrease in the number of cells releasing virus. Table 12 also indicates that the ability of the medium of BVPI mosquito cells to inhibit virus production was abolished by prior treatment with anti-Banzi viral serum but not with anti-mosquito cell serum.

13. Properties of the antiviral factor. Studies have shown that (i) the ability of medium of BVPI mosquito cells to inhibit Banzi viral replication is lost in a linear and progressive manner upon dilution, (ii) the antiviral factor inhibits the replication of Banzi virus and not of heterologous viruses (Table 13), (iii) the antiviral factor inhibits the replication of Banzi virus in mammalian cells as well as mosquito cells (Table 14), (iv) the antiviral activity is destroyed by treatment with protease K, but not by RNase or DNase and, (v) the antiviral activity is routinely detected in culture fluids after 2 days of infection; occassionally, it can be detected after 24 hours of infection (Figure 5).

14. Purification and Identification of the anti-viral factor. Preliminary experiments have indicated that anti-viral serum which had been treated with purified V-1 did not inactivate the antiviral factor present in culture medium of BVPI cells. These results suggested that the antiviral factor and V-1 have common antigenic sites and that V-1 and the antiviral factor may be similar.

Therefore, the fraction of culture medium of BVPI mosquito cells which contained the inhibitory factor was examined for the presence of V-1. In these experiments, culture medium of BVPI mosquito cells which had been labeled with [^{3}H] amino acids for 48 hours was collected, clarified and subjected to filtration through an Amicon UM-10 membrane filter. The filtrate which inhibited Banzi virus replication by 99.4% was subjected to electrophoresis on SDS-10% polyacrylamide gels. As controls, culture medium of uninfected mosquito cells was treated in an identical manner. Figure 6A shows that the one

radiolabeled peptide which was present in filtrates of medium of BVPI mosquito cells comigrated with V-1 of Banzi virus. However, filtrates of uninfected mosquito cells contained a protein which comigrated with V-1 (Figure 6B).

To demonstrate that the peak of radioactivity present in filtrates of BVPI mosquito cells (see Figure 6A) consists of a Banzi viral protein, as well as of a mosquito cell protein, filtrates of media were treated with either antiviral serum or anti-mosquito cell serum. As shown in Table 15, antiviral serum precipitated approximately 55% of radioactivity present in filtrates of medium of BVPI mosquito cells, but did not precipitate any labeled proteins from filtrates of medium of uninfected mosquito cells. Anti-mosquito cell serum precipitated radioactivity from filtrates of both culture media. These results suggest that filtrates of medium of BVPI mosquito cells contain two proteins of similar molecular weights, one of which is a cellular protein. The other protein appears to be of viral origin.

To separate the cellular protein and the viral protein, filtrates of culture medium of either BVPI mosquito cells or uninfected mosquito cells were subjected to high voltage paper electrophoresis (Figure 7). After electrophoresis of filtrates of medium of BVPI mosquito cells, two peaks of radioactivity were detected (Figure 7A). Only peak II was detectable in electropherograms of filtrates of medium of uninfected mosquito cells (Figure 7B). Panel C showed that V-1 isolated from purified virions exhibited the same mobility as peak I. Peaks I and II were eluted from the paper strip and then were incubated with either antiviral serum or anti-mosquito cell serum. Peak I reacted only with anti-viral serum whereas peak II reacted only with anti-mosquito cell serum (data not shown).

15. Similarities of V-1 and the viral protein in medium of BVPI mosquito cells. The viral protein in filtrates of medium of BVPI mosquito cell was eluted from paper strips after high-voltage electrophoresis and incubated with trypsin. The resulting tryptic peptides were resolved by high voltage paper electrophoresis at pH 3.5. V-1 was likewise eluted from paper strips and processed in a identical manner. Figure 8B shows that the tryptic peptides of V-1 had the same mobilities as those peptides generated by trypsin treatment of the viral protein found in filtrates of medium of BVPI mosquito cells (Figure 8A). Tryptic peptide map of the cellular protein present in filtrates of medium of BVPI mosquito cells (Peak II, Figure 7A) revealed no similarity to that of V-1 (data not shown).

Radioimmune competition experiments indicated immunological similarities between V-1 found in Banzi virions and the viral protein present in filtrates of medium of BVPI mosquito cells (Figure 9). Figure 9A shows that unlabeled, purified V-1 competes with [^{3}H] protein-labeled viral proteins in filtrates of medium of BVPI mosquito cells for antibodies against Banzi virus. Viral proteins in filtrates of medium of BVPI mosquito cells partially prevented the precipitation of labeled V-1 by anti-Banzi virus serum (Figure 9B).

Finally, the ability of V-1 isolated from Banzi virus to inhibit viral replication in mosquito cells was examined. Table 16 shows that viral protein eluted from paper strips after electrophoresis of filtrates of medium of BVPI mosquito cells inhibited the growth of Banzi virus by 99.6%. In contrast, the cellular protein isolated from filtrates of medium of BVPI mosquito cells had no effect on Banzi virus replication. Treatment of mosquito cells with V-1 isolated from purified Banzi virions resulted in a substantial decrease in the yield of progeny virions. Moreover, treatment of mosquito cells with V-1 isolated from Banzi virions did not affect the replication of Japanese encephalitis virus or of Eastern equine encephalitis virus (data not shown).

C. Conclusions

1) Cellular proteins are associated with purified Banzi virus grown in mosquito cells. These host cell proteins are glycoproteins and appear to be identical to those proteins comprising the naturally occurring, mosquito cell hemagglutinin.

2) Upon prolonged growth in mosquito cells, Banzi virus undergoes the following apparent changes: (i) a shift in the pH optimum of hemagglutination by Banzi virus, (ii) an increase of one cellular protein associated with Banzi virions and, (iii) increased reactivity of Banzi virus with anti-mosquito cell serum. However, other viruses, e.g., Dengue II virus and Sindbis virus did not react with anti-mosquito cell serum.

3) As a result of the incorporation of mosquito cell proteins into Banzi virions, mice immunized with uninfected mosquito cells are protected against infection (death) by Banzi virus propagated in mosquito cells. This protection is not due to any component of the medium and is due, in part, to the humoral response of the mouse to mosquito cell proteins. Since immunization of mice with purified A. albopictus cell hemagglutinin also protects mice from infection by mosquito cell grown virus, it is likely that the mosquito cell antigen which induces protective antibodies is one or more of the proteins constituting the A. albopictus hemagglutinin. The protective effect afforded mice by prior immunization with mosquito cells is also observed after challenged with other togaviruses, but not with bunya- or rhabdo- viruses. Furthermore, immunization of mice with a monoclonal cell line of A. albopictus cells also afforded protection against viral infection.

4) Accompanying the changes in the protein composition of the virus and in its immunological reactivities is the progressive appearance of an antiviral factor in the medium of Banzi virus-infected cells. This antiviral factor appears to be of viral origin, inhibits Banzi viral replication, and decreases the number of cells producing the virus. Furthermore, the antiviral agent inhibits the replication of only Banzi virus; no inhibitory effect on the replication of other togaviruses could be detected. This antiviral factor has been purified and identified by tryptic peptide mapping and by immunological techniques as being very similar, if not identical, to V-1 found in purified Banzi virions.

5) The temporal relationship between the changes in the structure of Banzi virus, in the production of a soluble viral protein able to inhibit viral replication, and in the evolution of acutely infected mosquito cells into a population of persistently infected mosquito cells suggests that the incorporation of host cell proteins into Banzi virus may be related to the establishment of a persistent state of infection.

PUBLICATIONS

1. Lee, C.-H., and R.H. Schloemer. 1981. Mosquito cells infected with Banzi virus secrete an antiviral activity which is of viral origin. Virol. 110 In press.

2. Lee, C.-H., and R.H. Schloemer. 1981. Identification of the antiviral factor in culture medium of mosquito cells persistently infected with Banzi virus. Virol. 110 In press.

Figure 1 Distribution of Banzi virus grown in BHK-21 cells or in A. albopictus cells in equilibrium density gradients. Banzi virus was grown in BHK-21 cells and A. albopictus cells in the presence of [^{35}] methionine and 100 mM excess NaCl. An equivalent number of A. albopictus cells was mock-infected and processed in an identical manner. After incubation, the culture medium was clarified by centrifugation at 1500 x g for 15 minutes, and virus was precipitated by polyethylene glycol. The resulting precipitate was resuspended and layered onto a 28 to 50% sucrose gradient. After centrifugation for 40 hours at 76,000 x g, gradients were fractionated into 1-ml aliquots and the presence of virus was determined by plaque assays on monolayers of BHK-21 cells. Location of the mosquito cell hemagglutinin was determined by HA assays at pH 5.8. Approximately 10^{13} PFU of virus were placed on the gradients. (A), Banzi virus grown in BHK-21 cells; (B), Banzi virus grown in A. albopictus cells; (C), medium from uninfected A. albopictus cells.

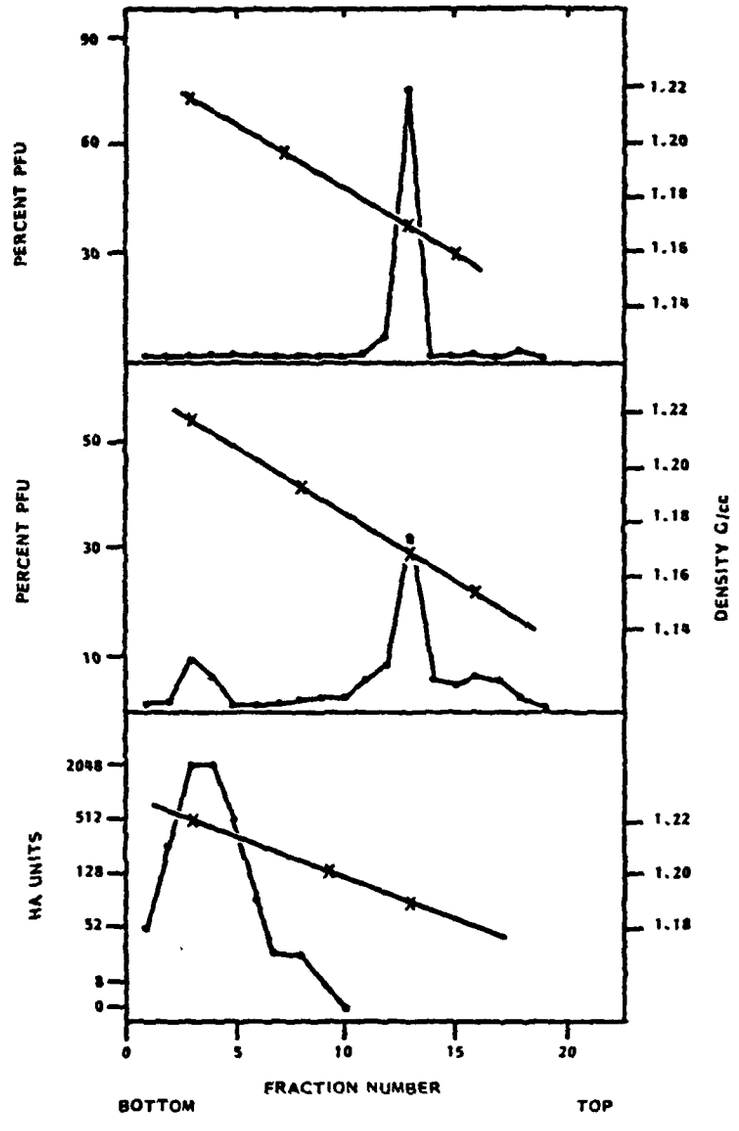
PERCENT PFU
90
60
30
1.22
1.20
1.18
1.16
1.14
PERCENT PFU
50
30
10
1.22
1.20
1.18
1.16
1.14
DENSITY G/cc
HA UNITS
2048
512
128
52
8
0
1.22
1.20
1.18
0
5
10
15
20
FRACTION NUMBER
BOTTOM
TOP

Fig. 1

Figure 2 Electropherograms of polypeptides of Banzi virus. Banzi virus grown in the presence of [^{35}S] methionine was purified by equilibrium gradient centrifugation as described in the legend of figure 1. Regions of gradients corresponding to a density of 1.17 g/cc were pooled and virus was precipitated by trichloroacetic acid. The mosquito cell hemagglutinin which banded at densities of 1.21 to 1.22 g/cc (see Figure 1C) was pooled and treated in a similar manner. (A), peptides of Banzi virus grown in BHK-21 cells; (B), peptides of Banzi virus propagated in A. albopictus cells; (C), peptides of mosquito cell hemagglutinin obtained from uninfected A. albopictus cells.

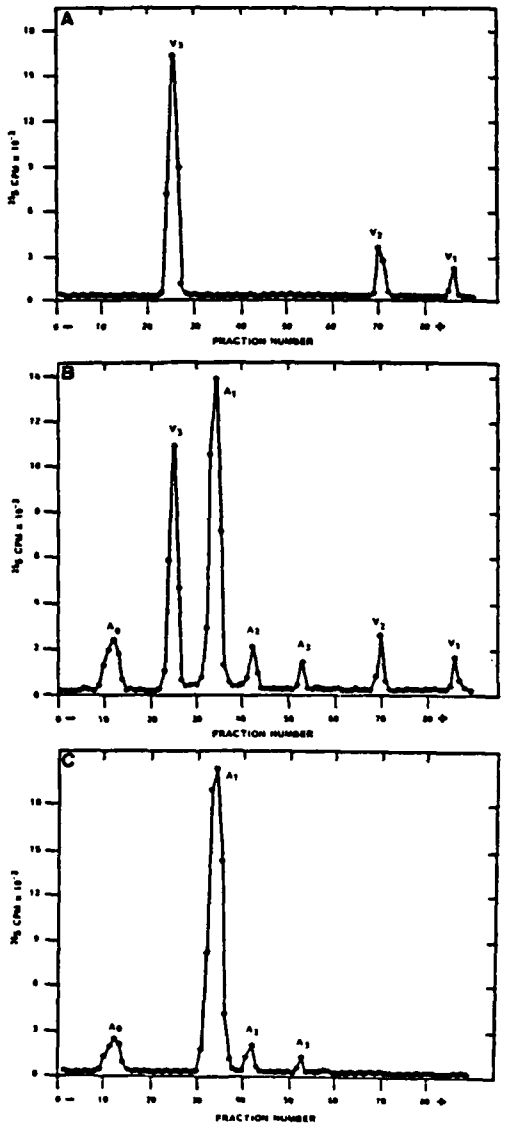

Fig. 2

Table 1 Summary of molecular weights of proteins found in purified Banzi virus grown in various cell lines[a]

Polypeptide	BV_{BHK} (daltons)	$BV_{A.albo}$ (daltons)	A. albopictus hemagglutinin (daltons)
V_3	56,000	53,000	ND
V_2	14,500	14,500	ND
V_1	8,400	8,400	ND
A_0	ND	88,000	88,000
A_1	ND	44,000	44,000
A_2	ND	35,000	35,000
A_3	ND	23,000	23,000

[a]Molecular weights of peptides were determined by comparison of their electrophoretic mobilities to those of bovine serum albumin, chicken avalbumin, trypsin and cytochrome C on 8.5% and 10% SDS-polyacrylamide gels. ND, not detected.

Figure 3 Electrophoretic profiles of polypeptides of Banzi virus precipitated by anti-Banzi viral serum or by antiserum to A. albopictus cells. Purified [^{35}S] methionine labeled Banzi virus grown in A. albopictus was dissociated with NP-^{40} and deoxycholate before addition of antiserum. Immunoprecipitation and electrophoresis was performed as described. Position of proteins was determined by electrophoresis in parallel gels of labeled Banzi virus. (A), polypeptides precipitated by antiviral serum; (B), polypeptides precipitated by antiserum to uninfected mosquito cells.

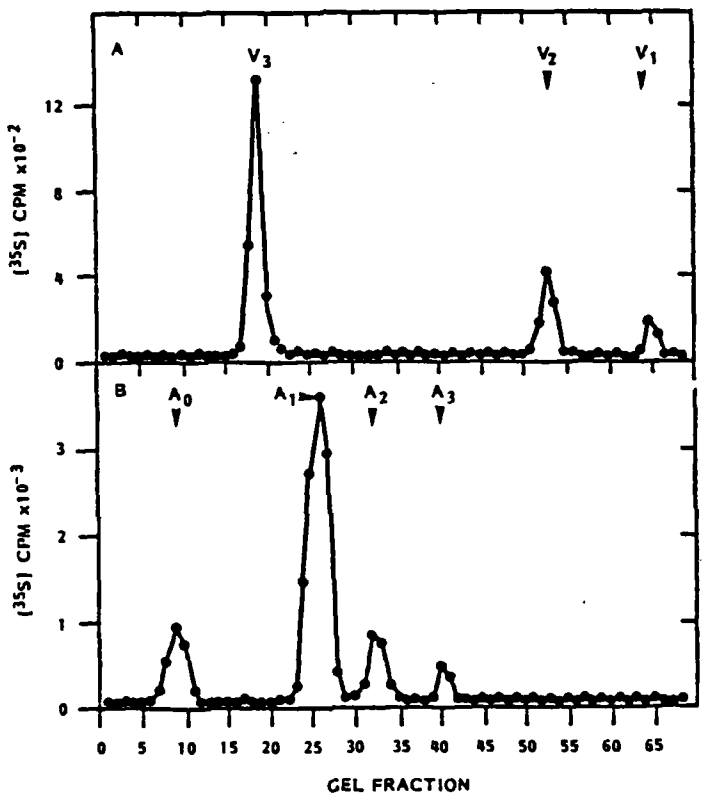
A
V_3
V_2
V_1
12
8
4
0
[^{35}S] CPM x10^{-2}
B
A_0
A_1
A_2
A_3
3
2
1
0
[^{35}S] CPM x10^{-3}
0 5 10 15 20 25 30 35 40 45 50 55 60 65
GEL FRACTION

Fig. 3

Table 2 Ability of antisera to precipitate intact Banzi virus

Labeled components of mixture	% [^{35}S] cpm in aggregates formed after incubation with[c]	
	anti-Banzi viral serum	anti-mosquito cell serum
Banzi virus$_{BHK}$[a]	55	<1
Banzi virus$_{A.albo}$[a]	59	45
A. albopictus hemagglutinin[b]	<1	53
A. albopictus hemagglutinin + unlabeled Banzi virus$_{BHK}$	<1	57
A. albopictus hemagglutinin + unlabeled Banzi virus$_{A.albo}$	<1	54
Banzi virus$_{BHK}$ + labeled A. albopictus hemagglutinin	56	<1

[a] [^{35}S] methionine labeled Banzi virus grown in either BHK-21 cells or A. albopictus cells for 24 hours were purified by equilibrium density centrifugation. Virions present in regions of gradients corresponding to a density of 1.17 g/cc were pooled, diluted and centrifuged at 76,000 x g for 3.5 hours onto a pad of 100% glycerol. Virus was diluted so that 2 x 10^7 PFU were present in 0.5 ml of PBS.

[b] A. albopictus hemagglutinin obtained from uninfected mosquito cells which had been labeled with [^{35}S] methionine was purified and diluted to a final concentration of approximately 32,000 HA units per 0.5 ml of PBS.

[c] Virus and A. albopictus hemagglutinin were incubated with various antisera at 4°C for 30 minutes and immune aggregates which formed were collected and assayed for [^{35}S] radioactivity. Results are expressed as the percent of total radioactivity originally present in each assay.

Table 3 Proteins associated with Banzi virus grown in mosquito cells for 1, 7 and 60 days[a]

Polypeptides	Relative amounts of proteins in Banzi virus grown in mosquito cells for following number of days		
	1	7	60
V_3	4.23	2.51	ND
V_2	1.0	1.0	1.0
V_1	0.80	0.81	0.77
A_0	0.92	0.95	0.9
A_1	5.54	7.01	8.96
A_2	0.92	1.01	0.95
A_3	1.0	0.8	1.0

[a]Banzi virus which had been grown in mosquito cells for 1, 7 or 60 days in the presence of $[^{35}S]$ methionine were purified as described in the text. The amount of $[^{35}S]$ methionine radioactivity in each peptide was determined after electrophoresis of disrupted virions on 8.5% polyacrylamide gels. Values were normalized to the amount of V_2 present in each virus preparation. ND, not detected.

Figure 4 Variation in pH optima for Banzi viral hemagglutination. Banzi virus were grown in A. albopictus cells for 24 hours (A), seven days (B) or for 12 days. Virions were purified as described in the legend to Figure 1. Virions which banded at a density of 1.17 g/cc were collected, dialyzed and precipitated by polyethylene glycol 6000. The resulting pellet was resuspended and used in HA assays performed at the indicated pH.

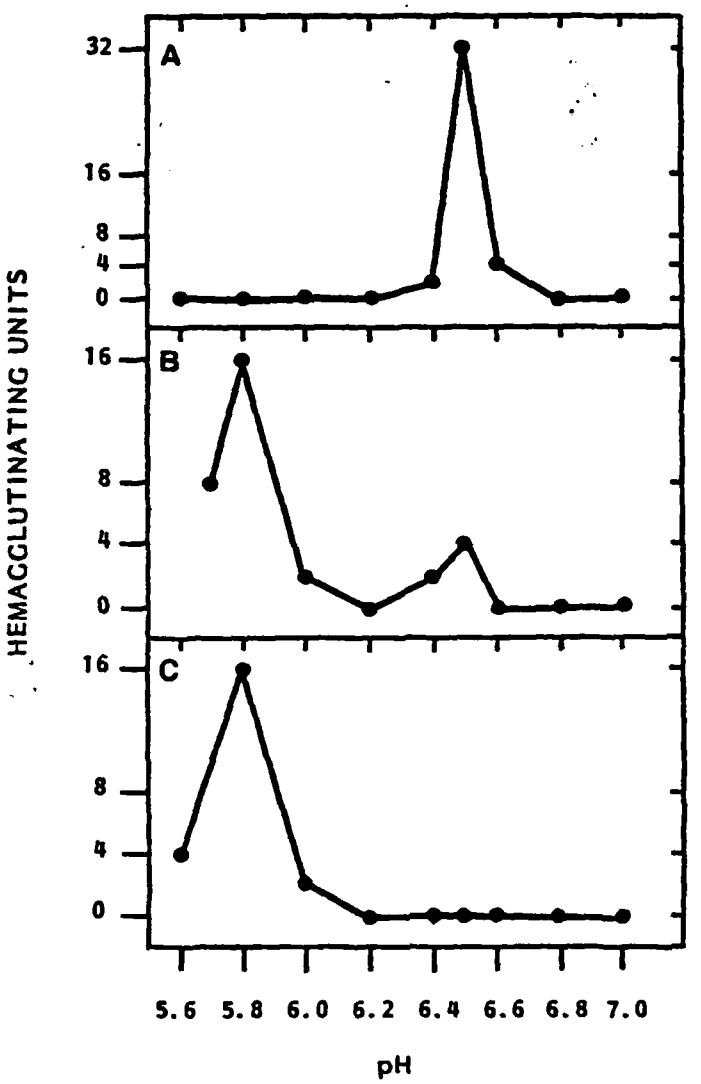
HEMAGGLUTINATING UNITS
32
16
8
4
0
A
16
8
4
0
B
16
8
4
0
C
5.6
5.8
6.0
6.2
6.4
6.6
6.8
7.0
pH

Fig. 4

Table 4 Effect of various antisera on hemagglutinating activity of purified Banzi virus propagated in various cell lines[a]

Serum	HAI titer against virus grown in				
	BHK	A.al (1)	A.al (7)		A.al(7)→BHK
			pH 6.5	pH 5.8	
Anti-Banzi virus	512	8	8	8	1024
Anti-BHK cell	4	4	4	4	4
Anti-A. albopictus cell	4	4096	8192	4096	4
Preimmune	4	4	4	4	4

[a]Virus grown in A. albopictus cells and BHK cells were purified as described in legend to Figure 1. Virions (ρ=1.17 g/cc) were collected, pooled and used in HAI assays. Anti-A. albopictus cell serum was prepared in rabbits as described in the text. Antiserum against BHK-21 cells was prepared in an identical manner whereas, anti-Banzi viral serum was prepared by immunization of rabbits with purified virus propagated in Vero cells. All sera were absorbed with fetal calf serum and complement was destroyed by heating at 56C for 30 minutes. HAI titer is calculated as the reciprocal of the highest dilution of serum which completely inhibited 8 HA units of Banzi virus at pH 6.5 when virus propagated in mosquito cells for 7 days were employed. ~~In this instance~~ HAI assays were conducted at pH 6.5 and pH 5.8 (final pH) since Banzi virus grown for 7 days in mosquito cells appear to have to pH optimum (see Figure 4B).

Table 5 Effect of various treatments of anti-mosquito cell serum on hemagglutinating activity of Banzi virus grown in mosquito cells for 24 hours.

Treatment of serum	HAI Titer[d]
None	1024
Preadsorbed with mosquito cells[a]	4
Preadsorbed with BHK-21 cells[a]	1024
Preadsorbed with Banzi virus[a] infected BHK-21 cells	1024
Preincubated with purified[b] mosquito cell hemagglutinin	16
Preincubated with trypsinized[c] mosquito cell hemagglutinin	512
Preincubated with trypsin and soybean trypsin inhibitor	1024

[a]Two ml of anti-mosquito cell serum were mixed with one ml of 10^7 uninfected A. albopictus cells, of 10^7 uninfected BHK cells, or of 10^7 Banzi virus-infected BHK cells. After incubation at room temperature for 30 minutes the mixtures were subjected to centrifugation 800 x g for 15 minutes to remove cells. The procedure was repeated once more. A sample of anti-mosquito cell serum received sufficient PBS to ensure that final volumes of all sera were equivalent. When 2×10^7 Banzi virus-infected BHK cells was washed with two ml of PBS for 1.5 hours, the amount of virus released from these cells was insufficient to cause agglutination of goose erythrocytes.

[b]Two ml of anti-mosquito cell serum were mixed with 0.5 ml of purified A. albopictus hemagglutinin (500 µg of protein). After incubation at room temperature for 30 minutes, the mixture was stored for 16 hours at 4°C. Immune precipities which had formed were removed by centrifugation. This procedure was repeated twice. Previous experiments had shown that anti-mosquito cell serum precipitates the proteins comprising the A. albopictus hemagglutinin. As a control, 0.5 ml of PBS was used instead of the purified A. albopictus hemagglutinin. The HAI of this control was 1024 and is not included in Table 5.

[c]Mosquito cell hemagglutinin was treated with trypsin (1 mg) for 2 hours at 37°C prior to use. Reaction was terminated by the addition of soybean trypsin inhibitor.

[d]All assays were performed at pH 6.5.

Table 6 Ability of anti-mosquito cell serum to neutralize infectivity of purified Banzi virus[a]

Serum	Neutralization titer of sera against virus grown in			
	BHK	A.al(1)	A.al(7)	A.al(7)→BHK(2)
Anti-Banzi virus	1024	512	512	1024
Anti-mosquito	4	16(8)	256	4
Anti-BHK cell	4	4	2	4
Preimmune	4	2	4	4

[a]Source of virus is described in Table 4. Neutralization tests were performed by mixing 200 PFU of Banzi virus in 0.2 ml of lactalbumin hydrolysate with 0.2 ml of two-fold serial dilution of various sera. After incubation at room temperature for 30 minutes, 0.2 ml of the mixture was plated on monolayers of BHK-21 cells. Three days after incubation at 37°C, the monolayers were stained with neutral red and plaques were scored. Anti-Banzi viral serum was initially diluted 1:5 before use.

Table 7 Resistance to challenge with Banzi virus induced by prior immunization of mice with A. albopictus cells[a]

Virus dose (LD_{50})	Survival after 21 days No. of mice/total	
	Unimmunized	Immunized
1	3/10	22/22
10	0/10	24/25
100	0/10	21/24

[a]Groups of mice (10-14 days old) were immunized with 10^7 disrupted A. albopictus cells i.p. once a week for five weeks. Unimmunized mice were injected with PBS. Thirty-five days after the initial injection, immunized and unimmunized mice were injected i.p. with various doses of Banzi virus grown seven days in A. albopictus cells. Virus innocula was back titrated at the time of the experiment.

Table 8 Ability of various immunogens to protect mice from challenge with Banzi virus[a]

Immunogen	Source of virus used as challenge	Survival of mice 21 days after challenge No. of mice/total	
		Expt 1	Expt 2
None	A.al	0/5	1/20
	BHK	0/5	0/20
A. albopictus cells	A.al	15/16	28/30
	BHK	0/16	0/20
M and M medium	A.al	0/9	1/25
	BHK	0/7	0/25
BHK-21 cells	A.al	0/15	0/10
	BHK	0/13	1/10
A. albopictus hemagglutinin	A.al	19/20	30/30
	BHK	0/20	2/30

[a]Mice (10-14 days old) were immunized i.p. with 10^7 disrupted A. albopictus cells or BHK-21 cells, with 0.5 ml of PBS (control), with M and M medium or with 100 µg of A. albopictus hemagglutinin which was purified as described in the text. Injections were given on day 0, 7, 21. On day 27, 100 LD_{50} of virus which was grown seven days in mosquito cells or in BHK-21 cells (BHK) were injected i.p. Virus innocula was back titrated at time of experiment.

Table 9 Survival of mice immunized with mosquito cells or with BHK cells after challenge with arboviruses[a]

Challenge virus	Cell line for viral growth	Cell line used as immunogen	No. of survivors/total (%) Expt 1	Expt 2
Eastern equine encephalitis virus	A. albopictus	A. albopictus	16/20 (80)	15/19 (79)
		BHK-21	0/10 (0)	0/5 (0)
	BHK-21	A. albopictus	0/14 (0)	0/20 (0)
		BHK-21	0/12 (0)	0/15 (0)
Germiston virus	A. albopictus	A. albopictus	0/20 (0)	0/10 (0)
		BHK-21	0/10 (0)	0/15 (0)
	BHK-21	A. albopictus	0/10 (0)	0/10 (0)
		BHK-21	0/10 (0)	0/10 (0)
Japanese encephalitis virus	A. albopictus	A. albopictus	26/30 (87)	13/15 (87)
		BHK-21	1/20 (5)	0/15 (0)
	BHK-21	A. albopictus	0/15 (0)	1/15 (6)
		BHK-21	0/15 (0)	0/15 (0)
Vesicular stomatitis	A. albopictus	A. albopictus	2/30 (7)	
		BHK-21	1/20 (5)	
	BHK-21	A. albopictus	0/20 (0)	
		BHK-21	1/15 (7)	

[a]The various viruses grown for 24 hours in BHK-21 cells or for 5 days in A. albopictus cells. Immunization of mice with cell lines is described in Table 8. Mice were challenged with 10 LD_{50} (back titrated at the time of experiment) and held for 21 days after viral challenge.

Table 10 Survival of mice immunized with monoclonal A. albopictus cells after injection with Banzi virus[a]

Cell line for viral growth	Immunogen	No. of survivors/total (%)
BHK-21	BHK-21 cells	0/10 (0)
	Dulbecco's medium	1/10 (10)
	A. albopictus cells	1/10 (10)
A. albopictus	BHK-21 cells	0/20 (0)
	Dulbecco's medium	1/10 (10)
	A. albopictus	29/30 (97)

[a]The experimental protocol is as described in Table 8, except that the monoclonal A. albopictus cell line was used. Mice were challenged with 10 LD_{50} of virus grown in mosquito cells for 7 days or in BHK-21 cells for 24 hours. The number of surviving mice after 21 days post-challenge is given.

Table 11 Growth of togaviruses in A. albopictus cells infected with Banzi virus[a]

Virus	Mosquito cells	Yield after 24 hr (PFU/ml)
None	Fresh	0
	Infected	5.2×10^4
Banzi virus	Fresh	1.2×10^9
	Infected	6.1×10^4
Japanese encephalitis virus	Fresh	6.8×10^7
	Infected	5.0×10^7
Eastern equine encephalitis virus	Fresh	2.5×10^8
	Infected	1.8×10^8

[a]Uninfected (fresh) mosquito cells or mosquito cells infected with Banzi virus for 90 days (infected) were infected with the indicated virus at a M.O.I. of 50 PFU/cell. After 24 hr of incubation at 28°, the yield of virus was determined by plaque assays on BHK-21 cells.

Table 12 Effects of medium of BVPI mosquito cell cultures on the replication of Banzi virus in mosquito cells[a]

Test substance used to treat mosquito cells prior to infection	Pretreatment of test substance	Virus yield		Infectious centers (%)
		PFU/ml	%	
None	None	8.7×10^8	100	115
Medium of uninfected mosquito cells	None	8.8×10^8	101	109
Medium of acutely infected mosquito cells	None	8.4×10^8	97	95
Medium of BVPI mosquito cells	None	3.3×10^8	0.4	5
Medium of BVPI mosquito cells	Incubation with antiviral serum	8.4×10^8	97	89
Medium of BVPI mosquito cells	Incubation with anti-mosquito cell serum	2.9×10^6	0.3	4

[a]Media of uninfected mosquito cells, of mosquito cells infected with Banzi virus for 24 hr (acute infection), or of BVPI mosquito cells were clarified by centrifugation at 800 g for 30 min and then were dialyzed against 2 volumes of M and M medium for 18 hr at 4°. Antiserum was incubated with medium of BVPI mosquito cells for 2 hr at 37° prior to dialysis. The resulting diffusates were sterilized by membrane filtration and 5 ml of each diffusate were incubated with uninfected mosquito cells for 24 hr prior to infection with Banzi virus (M.O.I. = 50 PFU/cell). After 24 hr of infection, the amount of extracellular virus was determined. In a separate experiment, the number of cells releasing virus after 24 hr of infection was determined by infectious center assays.

Table 13 Effect of antiviral agent on replication of togaviruses in mosquito cells[a]

Virus	Yield of virus (PFU/ml) from mosquito cells pretreated with diffusates of medium of	
	Uninfected mosquito cells	BVPI mosquito cells
Banzi virus	1.2×10^9	7.2×10^6
Japanese encephalitis virus	6.9×10^8	6.5×10^8
Eastern equine encephalitis virus	8.1×10^8	8.5×10^8

[a]Uninfected mosquito cells were treated for 24 hr with diffusates of medium of cultures either of uninfected mosquito cells or of BVPI mosquito cells as described in Table 12. Cells were infected with the indicated virus at an M.O.I. of 50 PFU/cell and then incubated for 24 hr at 28°. The amount of virus in the medium was determined by plaque assays.

Table 14 Effect of anti-Banzi viral factor on Banzi virus replication on BHK-21 cells[a]

Test substances used to treat BHK-21 cells prior to infection	Pretreatment of test substance	Virus yield	
		PFU/ml	%
M and M medium	None	9.4×10^7	100
Medium of uninfected mosquito cells	None	9.2×10^7	98
Medium of BVPI mosquito cells	None	7.6×10^6	8.1
Medium of BVPI mosquito cells	Incubation with antiviral serum	9.7×10^7	103
Medium of BVPI mosquito cells	Incubation with anti-mosquito cell serum	5.8×10^6	6.1

[a]All media were dialyzed against 2 volumes of Dulbecco's modified medium for 18 hr at 4°. Treatments of medium of BVPI mosquito cells with antisera are described in Table 12. Monolayers of uninfected BHK cells were treated with 5 ml of the indicated diffusates for 24 hr at 37°. Cells were then infected with Banzi virus at a multiplicity of infection of 50 PFU/cell. The amount of extracellular virus present 24 hr postinfection was determined by plaque assays on BHK cells.

Figure 5 Time course of appearance of antiviral activity. Mosquito cells were infected with Banzi virus at a MOI of 50. At the indicated times medium was collected and assayed for the presence of antiviral activity as described in Table 12. Experiment was done in duplicate.

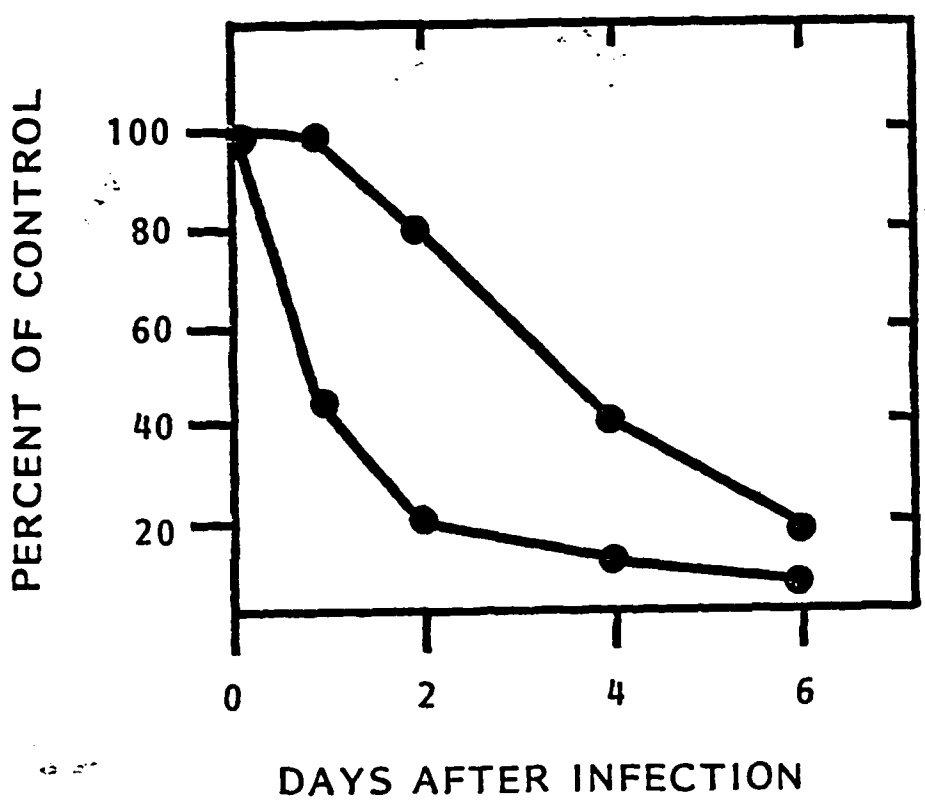

Fig. 5

Figure 6 Electropherogram of polypeptides present in filtrates of medium of BVPI mosquito cells. Monolayers of uninfected mosquito cells or of BVPI mosquito cells were labeled for 48 hours with ^{3}H-amino acids (10 µCi/ml). Culture medium was collected, clarified by centrifugation at 800 g for 15 minutes, and subjected to filtration through an Amicon UM-10 membrane filter. The filtrates were mixed with ^{14}C-protein-labeled Banzi virus grown in BHK-21 cells and subjected to electrophoresis on SDS-10% polyacrylamide gels. Panel A, electropherogram of filtrate of medium of BVPI mosquito cells; panel B, electropherogram of filtrate of medium of uninfected mosquito cells. Symbols: (0), ^{14}C; (●), ^{3}H.

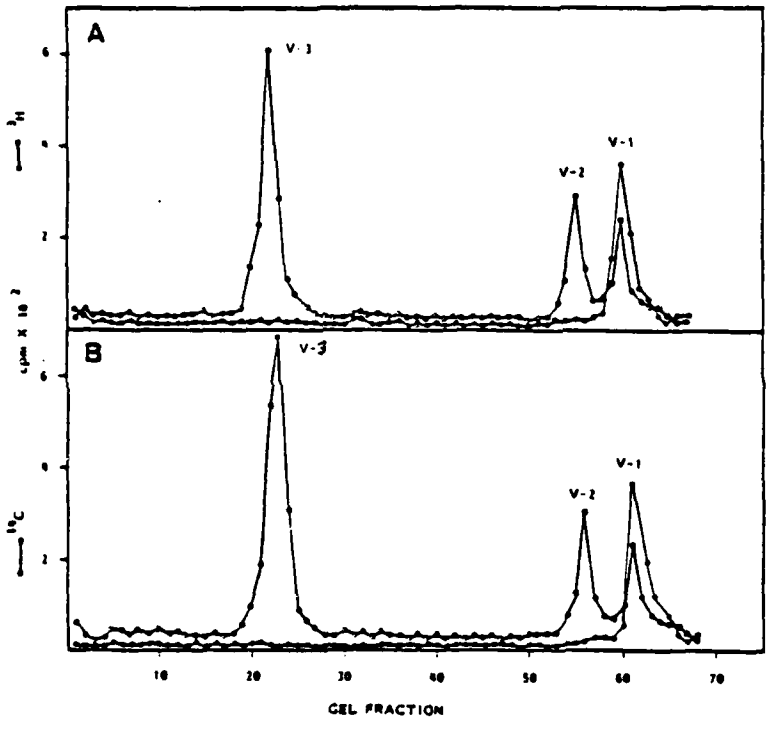

Fig. 6

Table 15 Reactivity of filtrates of medium of BVPI mosquito cells with various antisera[a]

Filtrates of medium from	^{3}H cpm precipitated by	
	Anti-Banzi virus serum	Anti-mosquito cell serum
Uninfected mosquito cells	83 (7.6)	793 (72.7)
BVPI mosquito cells	1071 (55.6)	668 (34.7)

[a]Monolayers of uninfected mosquito cells or of BVPI mosquito cells were labeled with ^{3}H-amino acids (10 µCi/ml) for 48 hr. The culture medium was clarified and filtered through an Amicon UM-10 membrane filter and the resulting filtrate was lyophilized and reconstituted in PBS. Aliquots (100 µl) were incubated with 100 µl of various antisera. The numbers in parentheses represent percentage of original radioactivity which was precipitated by the antiserum. Preimmune serum precipitated between 5.7 and 9.8% of the radioactivity.

Figure 7 Paper electrophoresis of filtrates of medium of BPVI mosquito cells. Filtrates of media of ^{3}H-protein-labeled BVPI mosquito cells and of uninfected mosquito cells were prepared as described in the legend to Figure 6 and 100-µl samples were subjected to high-voltage paper electrophoresis at pH 8.6. After electrophoresis, the paper was cut into 1-cm sections and assayed for radioactivity. For comparative purposes V-1 isolated from ^{3}H-protein-labeled Banzi virus was treated in an identical manner. Panels A and B, Pattern obtained from medium of BPVI mosquito cells and of uninfected mosquito cells, respectively; panel C, pattern obtained from V-1. Arrow indicates origin.

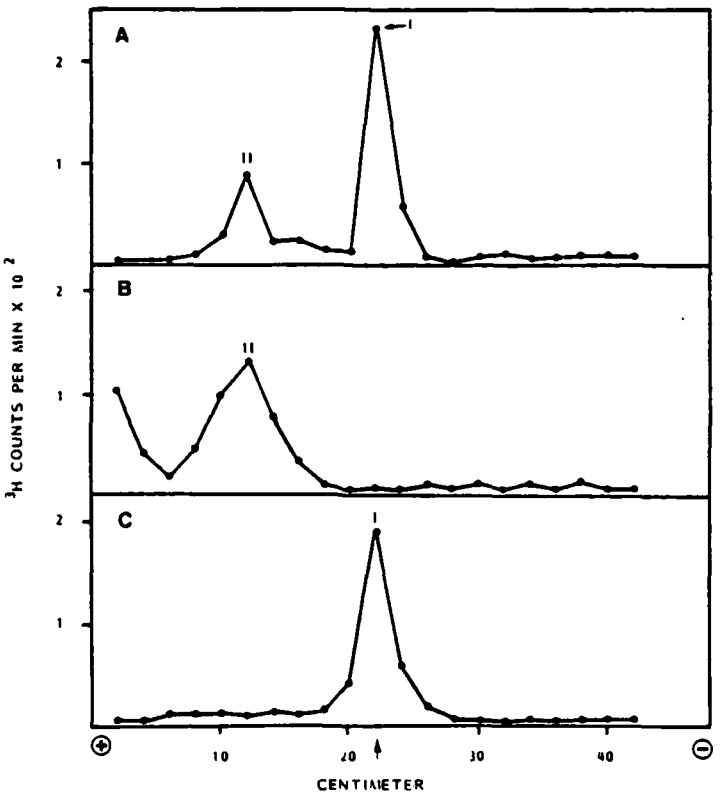

Fig. 7

Figure 8 Tryptic peptide analysis of viral protein present in medium of BVPI mosquito cells. The polypeptide which remained at the origin after paper electrophoresis of filtrates of medium of BVPI mosquito cells was eluted from paper strips similar to those shown in Figure 7A. Eluted polypeptides were digested with trypsin (100 µg) for 48 hours and the resulting tryptic peptides were resolved by paper electrophoresis at pH 3.5. After electrophoresis, the paper was cut into 1-cm sections and radioactivity present in each section was determined. Banzi virion V-1 was treated in an identical manner. Samples shown together were coelectrophoresed. Panel A, Tryptic peptides of viral protein in filtrates of medium of BVPI mosquito cells; panel B, tryptic peptides of V-1. Arrow indicates origin.

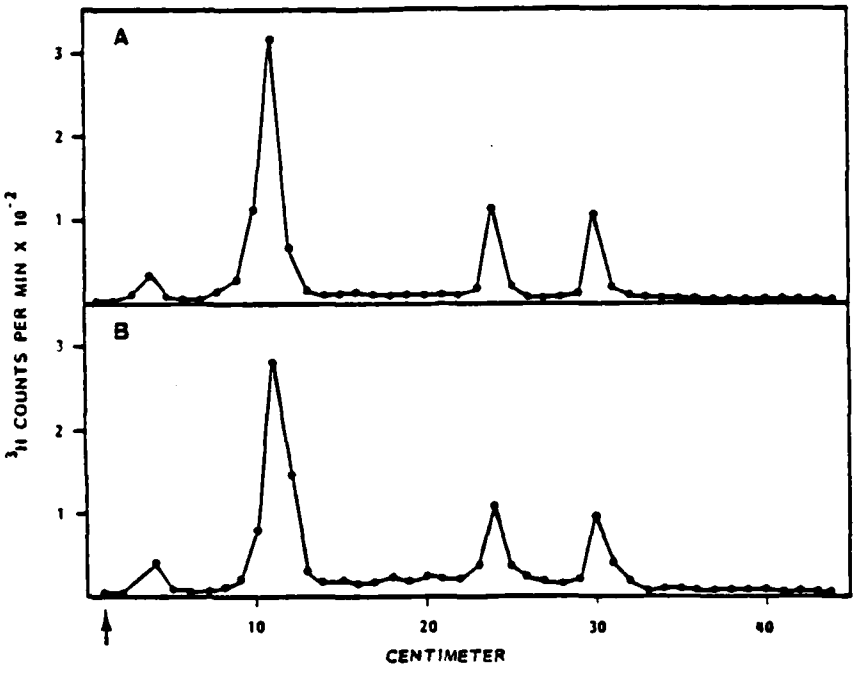

Fig. 8

Figure 9 Antigenic relatedness of V-1 and of the viral protein present in filtrates of medium of BVPI mosquito cells. Radioimmune competition experiments were conducted using either filtrates of medium of ^{3}H-protein-labeled BVPI mosquito cells and various amounts of unlabeled V-1 isolated from Banzi virus (Panel A) or ^{3}H-protein-labeled V-1 and various amounts of unlabeled filtrates of medium of BVPI mosquito cells (Panel B). Fifty-microliter aliquots of the radiolabeled polypeptide were incubated with 50 µl of anti-Banzi virus serum and the indicated amounts of unlabeled polypeptide in a total volume of 150 µl. After incubation at 18 hours at 4°C, 50 µl of goat anti-rabbit IgG was added. The mixtures were then placed at 4°C for 18 hr. After centrifugation at 10,000 x g for 20 min, the resulting precipitates were washed with PBS and assayed for radioactivity.

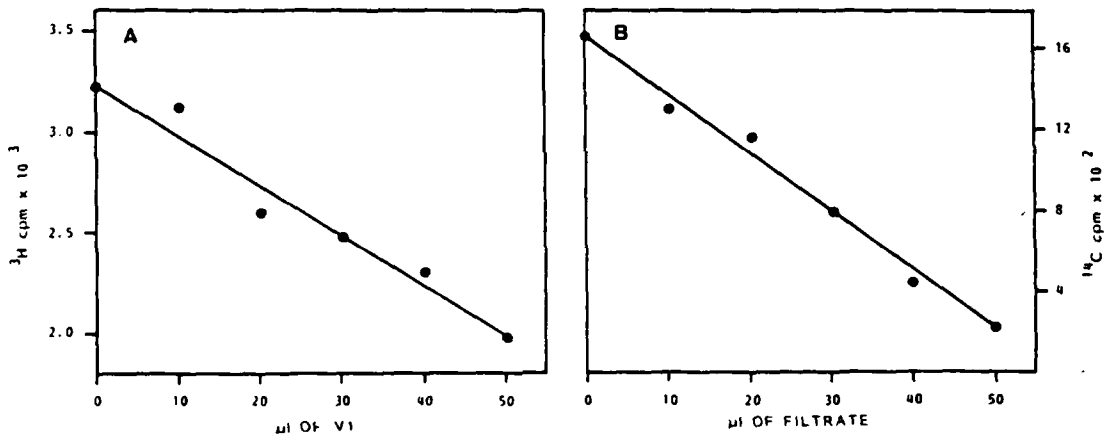

Fig. 9

Table 16 Ability of V-1 to inhibit Banzi virus replication[a]

Mosquito cells pretreated with	Virus yield	
	PFU/ml	%
PBS	7.4×10^8	100
V-1 isolated from BVPI mosquito cells	2.8×10^6	0.37
Cellular protein isolated from BVPI mosquito cells	6.0×10^8	81.1
V-1 isolated from Banzi virions	5.8×10^6	0.78

[a]Filtrate of medium of BVPI mosquito cells and purified V-1 isolated from Banzi virions were subjected to high-voltage paper electrophoresis as described in the legend to Fig. 7. Material which had remained at the origin of the electropherogram and material which had migrated 12 cm from the origin were eluted with PBS. Monolayers of mosquito cells were incubated for 8 hr at 28° with 2 ml of either V-1 (51 µg/ml) or the cellular protein (45 µg/ml) which had been isolated from culture medium of BVPI mosquito cells or with 2 ml of V-1 (205 µg/ml) isolated from purified Banzi virus. Treated cultures were then infected with Banzi virus (M.O.I. = 50 PFU/cell). The amount of extracellular virions present after 24 hr of incubation at 28° was determined and compared to the virus yield from mosquito cells which had been pretreated with PBS.

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