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The studies utilized our model system: the response of 2 congenic strains of histocompatible C3H mice to infection with Banzi virus. One strain (C3H/He) is highly susceptible to lethal infection with Banzi virus and the other strain (C3H/RV) is highly resistant to lethal infection with this virus. Experiments planned for the year were to examine the ultrastructural morphogenesis of viral encephalitis, evaluate a potential role for defective interfering particles in resistance to infection and extend characterization of immunological responses to Banzi virus. Comparisons of response between resistant and susceptible mice, in vitro or in vivo were to be stressed for each of the studies.

We requested that the contract be terminated on August 31, 1978, 2 months after funding began, because other longer term sources of funding became available. During the period from July 1 to August 31 work was begun on an ultrastructural pathogenesis study of Banzi virus infection in resistant and susceptible mice. We also continued efforts to refine the microcytoxicity test for cell mediated immune responsiveness to Banzi virus. Data developed during this period was not sufficient to draw additional conclusions about the phenotypic expression of genetic resistance.

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MECHANISM OF INNATE RESISTANCE TO FLAVIVIRAL ENCEPHALITIS

Final Report

Robert O. Jacoby and Pravin N. Bhatt

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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SUMMARY:

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities 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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I. Statement of Problem

Arthropod-borne (toga) viruses are a hazard to United States military personnel in many areas of the world. More specifically, neurotropic flaviviruses constitute a significant class of human pathogens and include: 1) Japanese B Encephalitis virus (Japan, Korea, Thailand, Vietnam and other regions of Southeast Asia); 2) Murray Valley Encephalitis virus (Australasia); 3) Russian Spring-Summer Encephalitis virus (central and eastern Europe and Asia); 4) West Nile virus (Africa, Middle East and Western Europe); 5) St. Louis Encephalitis virus (U.S.); 6) Powasan virus (U.S. and Canada).

All of these viruses can cause severe, and occasionally, fatal encephalitis. Yet, overt encephalitis is an uncommon sequella of flavivirus infection among people living in endemic areas. Studies with mumps and measles viruses have shown that mild or asymptomatic encephalitis accompanies a significant portion of human infections. Similar studies for flavivirus infection are not readily available. It is not clear why some individuals are able to deal effectively with neurotropic flaviviruses whereas others develop severe or fatal illness. Nevertheless, it seems reasonable to speculate that U.S. military personnel introduced to an endemic area would constitute a highly susceptible population and would experience a high rate of infection. In contrast, local inhabitants are more likely to be immune to infection from previous contact. Moreover, effective vaccination procedures for most neurotropic flavivirus infections have not yet been developed.

In our view, clarification of host defense mechanisms, including the role of genetic influences, could play a vital role in delineating the pathogenesis of arthropod-borne viral encephalitides. Such infromation could be applied toward: 1) predicting the clinical and epidemiological consequences of introducing susceptible personnel into endemic regions; 2) determining prognoses in infected individuals and, 3) developing a rational procedure for vaccination.

It should be emphasized that we view the Banzi virus model as an experimental prototype for neurotropic flaviviral encephalitis. The low pathogenicity of Banzi virus for laboratory personnel makes it much easier and safer to work with than some of the more pathogenic viruses in this group. Nevertheless, once the mechanisms of Banzi virus-host interactions have been clarified, we expect to extrapolate our findings to some of the more common human pathogens listed above.

II. Background

Resistance to viral infection can be acquired or innate. Acquired resistance results from priming of immunological defenses by previous encounters with a virus such as following naturally occurring infection or vaccination. In contrast, innate resistance is characterized by defiance to infection on initial encounter with a virus. It may be due to a total refractiveness of a species to viral penetration or replication (1), but in the context of this discussion, it refers to the ability of individuals to overcome infection with viruses which are lethal for a species as a whole.

It is well established that innate resistance to flaviviruses (formerly arbovirus group B) is inherited in Mendelian patterns as a simple autosomal

dominant trait (2, 4). Resistance is virus group-specific since mice resistant to lethal flaviviral infection are generally susceptible to lethal alpha virus (formerly arbovirus group A) infection (2). The adjectives "innate" and "genetic" are used interchangeably with respect to resistance throughout this text.

Genetic resistance to flaviviruses was first observed by Webster more than 40 years ago (3, 4), but the mechanisms which account for the phenotypic expression of resistance remain poorly understood. However, two major working hypotheses have evolved. The first proposes that cells from resistant mice cannot or do not support replication of infectious flaviviruses as well as cells from susceptible mice. In vitro studies have shown that peritoneal macrophages from strains of mice resistant to West Nile (WN) virus and the 17D strain of yellow fever (YF) virus yield less infectious virus than macrophages from susceptible mice (5, 6). Early work by Webster indicated that cultured brain fragments from resistant mice yielded less St. Louis encephalitis virus than brain fragments from susceptible mice (7). Recent evidence indicates that even embryonic fibroblasts from resistant mice yield less infectious virus than embryonic fibroblasts from susceptible mice (8). It has been suggested that this effect may be due to enhanced production of defective interfering (DI) particles in resistant cells (8).

The second theory proposes that genetic resistance is expressed phenotypically through the lymphoreticular system and implies that lymphoid cells from resistant mice are functionally superior to lymphoid cells from susceptible mice in defending against lethal flavivirus infection. Initial support for this theory came from demonstration that x-irradiation or radiominetic drugs abrogated genetic resistance to WN virus (5). The studies were not embellished by virological and histological characterizations of infected mice, so the effects of immunosuppression on the pathogenesis of disease remained unknown.

Complementary evidence came from extensive studies of genetic resistance to mouse hepatitis virus (MHV) carried out largely in the laboratory of Dr. Fred Bang (9-11). Resistance to MHV was also compromised by immunosuppressive drugs (12, 13) and Allison reported that resistance to MHV failed to develop in mice or their cultured macrophages after neonatal thymectomy (14). Schell showed that genetic resistance to ectromelia virus in C57/Bl mice may depend on the promptness of immunological responsiveness in some situations (15).

Goodman and Koprowski tried a more direct test of the "lymphoreticular cell" theory by making reciprocal transfers of nonimmune lymphoid cells between resistant and susceptible mice before challenging them with WN virus (6). They were able to partially protect susceptible mice from lethal encephalitis by giving them resistant lymphoid cells. They also presented evidence suggesting resistant mice given susceptible lymphoid cells were more susceptible to lethal viral infection. Unfortunately, they used allogenic mice for their experiments. Thus, graft-versus-host disease occurred in recipient mice and only small numbers of mice, whose chimeric status was not defined remained for infectivity studies.

The role of interferon in innate genetic resistance also is not clear. Vainio and colleagues (16) found that brain interferon levels to WN virus were higher in susceptible mice infected with WN virus than in infected resistance mice, perhaps because susceptible mice had more virus in brain. Hanson and others

were able to protect resistant mice against WN infection with lower concentrations of interferon then required to protect susceptible mice (17). Interferon did protect PRI and C3H/He mice equally well against Sindbis virus (alphavirus) to which they were equally susceptible.

The model system we are studying is Banzi virus infection in genetically resistant and genetically susceptible congenic mice. Breeding stock were originally obtained from Dr. Hillary Koprowski and a specific pathogen free colony was developed at Yale. The resistant strain is designated C3H/RV (RV) and the susceptible strain is C3H/He (He). They are histocompatible as determined by tumor grafting, serological assay (18) skin grafting and mixed leukocyte culture assay (19). Banzi virus was originally isolated from an African child with a febrile illness (20) and was a gift of Dr. Jordi Casals.

Our initial studies, which are detailed in reference (21) showed that Banzi virus was equally infectious for adult RV and He mice, but that it was about 100,000 times more lethal for He mice than RV mice following intraperitoneal (IP) inoculation. Banzi virus caused encephalitis in both strains, but RV mice were able to limit infection, remain asymptomatic, and recover, whereas He mice developed an encephalitic syndrome and died in 7 to 9 days. Virus replicated in tissues of both strains, but yields were significantly greater from He brains than from RV brains. Meningoencephalitis occurred in both strains, but neuronal necrosis was severe only in brains of He mice during the height of infection, but viral antigen was not detected in brains of RV mice. We also confirmed that genetic resistance of RV mice to lethal infection was virus group-specific since He and RV adults were essentially equally susceptible to alphavirus infection (Semliki forest (SF)) virus or Venezuelan equine encephalomyelitis (VEE) virus. Resistance to lethal Banzi virus infection in RV mice developed postnatally and did not reach significant levels until mice were 4 weeks old.

Resistance of adult RV mice to lethal infection could be compromised in several ways (22). First, intracerebral (IC) inoculation of virus produced high mortality in both strains. Virus titers, distribution of viral antigen and brain lesions (necrotizing meningoencephalitis) were also similar in both strains. Second, resistance of infected RV mice was also severely compromised by several types of immunological crippling: sublethal X-irradiation; cyclophosphamide (CY) treatment or T lymphocyte depletion (adult thymectomy followed by lethal x-irradiation and syngeneic bone marrow grafting or treatment with rabbit antimouse thymocyte serum (RAMTS). Paradoxically, T-cell depletion of He mice with RAMTS prolonged average survival time (AST) of Banzi virus-infected mice by several days compared to infected He mice given normal rabbit serum (NRS). CY treatment did not prolong survival of He mice. Virus yields, distribution of viral antigen and brain lesions among CY-treated RV mice were similar to those in non-immunosuppressed He mice. Virus yields and lesions in T-cell-depleted mice were similar to, but less severe than, those of infected CY-treated mice and the course of infection was longer. For example, virus titers peaked earlier and at higher levels, the spread of viral antigen was faster and more diffuse and the extent of neuronal necrosis was greater in CY-treated RV mice than in T-cell-depleted RV mice. In addition, CY-treated mice had no detectable HAI antibody titers comparable to nonimmunosuppressed control RV mice infected with Banzi virus. HAI titers were not reduced by T-cell depletion.

When AST among infected RV mice given various immunosuppressive treatments were compared, CY-treated mice always had a shorter AST than T-cell-depleted mice. Similarly, ASTs among RV mice compromised by several methods were always one to several days longer than for He mice infected the same way.

We also examined the responses of He and RV mice to immunization with formalinized Banzi virus vaccine (BV) or with vaccine incorporated into incomplete Freund's adjuvant (BV+IFA) or complete Freund's adjuvant (BV+CFA). RV mice primed IP with one dose of BV+CFA were protected from lethal IC challenge with virus by postvaccination day 6 whereas He mice primed with BV+CVA succumbed to ic challenge given up to 15 days post-priming. BV+IFA rendered RV mice only partially immune to IC challenge and BV alone did not protect. Viral replication in brains of vaccinated IC-challenged RV mice was low and encephalitis was mild and transient. In contrast, viral titers in vaccinated IC-challenged He mice were high and severe necrotizing chorio-meningoencephalitis developed. Viral antigen appeared in only small quantities in RV brain whereas antigen was widespread in He brains. He mice were able to survive IP or IV challenge when primed with any of the 3 vaccine preparations. He mice were also protected from IP challenge, but not IC challenge, by passive immunization with anti-Banzi immune ascitic fluid. Passively immunized RV mice were, however, protected from IC challenge with live virus. These preliminary studies together with our previous serological data indicate that both He and RV mice can respond to Banzi antigen, but more detailed kinetic studies of the primary response to virus are required to determine if differences critical to the outcome of infection exist. It should also be determined if the massive inflammation in vaccinated-challenged He mice has immunopathological components and if the differences in response to IC challenge can be explained by the slightly lower level of viral replication previously reported in unprimed RV compared to unprimed He mice (21 and unpublished data - see below).

Since the immunosuppression experiments cited above suggested that T-cells are required for full phenotypic expression of resistance, we began additional examination of T-cell particpation. First, we adapted an in vitro assay for cell mediated immunity (23) based on release of ^{51}Cr from target cells infected with Banzi virus after exposure to virus-primed spleen cells (24). The targets are L cells (mouse fibroblasts) which are derived from C3H mice and are, therefore, syngeneic with RV and He donor cells. Results showed that: 1) RV and He spleen cells from Banzi virus-primed donors are specifically cytotoxic for infected L cells by 6 days post-priming; 2) pretreatment of immune RV or He spleen cells with anti-thymocytes serum abolishes cytotoxic activity of spleen cells and 3) nylon passaged (T-cell enriched) spleen cells are as cytotoxic for target cells as unfractionated spleen cells. This assay is, to our knowledge, the first in vitro system for studying cell-mediated immune responses to flaviviruses. It has provided additional evidence for T-cell involvement in primary responses to Banzi virus and is considered again in the research plan.

Second, we began testing various adoptive immunization procedures prior to more detailed in vivo dissection of immunological defenses in genetic resistance. We first tried to confirm the previous work of Goodman and Koprowski with WN virus (6) by attempting to protect He mice from Lethal IP challenge with Banzi virus using non-immune donor spleen or lymph node (LN) cells. We also tried compromising resistance of RV mice by adoptive transfer of non-immune He spleen or lymph node cells. Various protocols were examined including: 1) transfer of up to 10^9 spleen or LN cells to unaltered recipients; 2) chal-

lenging recipients 1, 7 or 14 days after adoptive transfer; 3) more extensive repopulation of recipients with donor cells by single or repeated transfers of $2x10^8$ cells to thymectomized, lethally-irradiated (950R) recipients reconstituted with donor type bone marrow cells; 4) parabiosing RV and He mice where partners were unaltered or where 1 partner had been thymectomized, irradiated and reconstituted with congenic bone marrow prior to parabiosis. (Mice were surgically separated about 10 days before challenge). The upshot of these experiments was that He mice given non-immune RV donor spleen or LN cells frequently but not invariably, had ASTs several days longer than He mice given syngeneic He cells, dead RV cells or no cells. Best results were obtained in unaltered or sublethally irradiated recipient mice challenged 1 or 7 days after transfer. All attempts to render RV mice susceptible to lethal Banzi viral infection by injecting He lymphoid cells before challenge were unsuccessful.

More recently we have used donor cells from live virus-primed mice. With this procedure, primed RV donor cells conferred partial protection (increased AST) to complete protection from lethal IP challenge on He mice. Significant prolongation of AST in He recipients occurred after IV injection of RV spleen cells harvested as early as 6 days post-priming.

Adoptive immunization protocols have now been refined to establish optimum conditions for demonstrating the transfer of resistance. Experiments confirm that susceptible (He) mice can be protected from mortality after subcutaneous (SQ) challenge with virulent Banzi virus by adoptive transfer of virus-primed spleen cells.

Taken collectively, these data indicate that phenotypic expression of genetic resistance requires host factors independent of innate resistance of tissues to viral replication and that among these host factors immunological competence plays an important role.

In addition, these studies support the notion that cell-mediated immunity is an important host defense in experimental flavivirus infection. They also suggest that immune T-cells are required for adoptive transfer of protection and that immune C3H/RV spleen cells are, on a cell-for-cell basis, more efficient than immune C3H/He spleen cells at conferring adoptive immunity.

With respect to nonimmunological mechanisms in genetic resistance, we feel that the small, but potentially significant, differences in AST, LD_{50}, brain titers and lesions between IC-inoculated or immunosuppressed RV adults and untreated He adults observed in untreated He adults observed in our earlier work may be due to innate resistance of target cells or to some other nonimmunological events. In contrast to <u>in vitro</u> work with other flaviviruses discussed previously Banzi virus replicates equally well in target cells from resistant and susceptible mice. For example, virus yields from macrophage cultures or from cultures of infant or adult brain were essentially identical for He and RV mice. Nevertheless, closer examination of virus replication in brains during early stages of infection revealed that virus titers were persistently about one log lower in RV mice than in He mice.

On balance, genetic resistance to flaviviruses appears to be a multifactorial process. Our model system offers several advantages for studying this phenomenon. First, Banzi virus is highly infectious and, for He mice, highly lethal after parenteral challenge of adults. This is in contrast to several

other prototype flaviviruses used for this type of research since their virulence for adult mice after parenteral inoculation is low. Second, Banzi virus is adaptable for use in vitro assays of immunological phenomena. Third, mice used in this study are congenic so reciprocal cell transfer studies can be performed without danger of immunological rejection. Fourth, the system offers the possibility of exploring a non-H-2 linked genetically-determined immune response to an infectious agent.

III. Approach to the Problem

Phenotypic expression of genetic resistance to flaviviruses is probably a multifactorial phenomenon involving nonimmunological and immunological events. Recent work from our laboratory indicates that small persistent differences occur in the rate of viral replication in brains of susceptible mice compared to resistent mice during early stages of infection. Since it is unlikely that immune responses operate during this period, nonimmunological mechanisms involving virus-target cell interactions may be critical in determining the ability of the host to survive. On the other hand, we have also shown that immunological competence is required for full phenotypic expression of resistance. Both humoral and cell-mediated immune responses participate in host defenses to infection in resistant and susceptible mice. Preliminary studies of adoptive immunization in protection of mice against lethal Banzi viral infection suggest, however, that immune lymphoreticular cells from resistant mice are more efficient in conferring protection than cells from susceptible mice. Therefore, we feel that subtle differences between resistant and susceptible mice in 1) target cell susceptibility to viral infection or replication and in 2) the development and intensity of immunological responses to virus will determine the outcome of infection.

Comparison between immunological and nonimmunological defenses of resistant and susceptible mice, especially during early stages of infection should be a productive approach to thisproblem. Our model system is especially suitable for these studies since Banzi virus elicits large, reproducible differences in clinical signs, mortality, virus yields and lesions between resistant and susceptible mice after parenteral challenge.

The specific objectives of the current proposal were:

a.) to study nonimmune virus-host interactions, especially during early stages of infection by:

   1.) comparing the pathogenesis of infection, ultrastructurally, between resistant and susceptible mice.

   2.) preliminary evaluation of potential roles for DI particles and/or interferon in modulating viral replication in resistant and susceptible mice.

b.) to continue characterization of the role of the lymphoreticular system in the development and expression of genetic resistance by comparing, quantitatively, primary humoral immune responses and cell-mediated immune responses to Banzi virus between resistant and susceptible mice.

IV. Results of Experiments Performed From July 1, 1978 - August 31, 1978.

A. The pathogenesis of encephalitis caused by Banzi virus in resistant and susceptible mice as determined by electron microscopy.

Our previous studies indicated that small differences in viral titer persist from early stages of infection between brains of infected RV and He mice regardless of the route of inoculation. This suggests that non-immunological mechanisms contribute to control of viral replication in RV mice. In order to study potential morphological differences in the response of T cells from RV and He mice to Banzi virus, spleen, thymus and 3 areas of brain (olfactory bulb, frontal cortex and hippocampus) were harvested daily from parenterally-challenged mice for 2 weeks. Tissues were processed for electron microscopy, but they were not examined before the contract was terminated.

B. Refinement of a microcytotoxicity test for cell-mediated immunity to Banzi virus.

We had previously shown that splenic cells from RV or He infected with Banzi virus were cytotoxic for Banzi virus-infected L-929 fibroblasts as determined by ^{51}Cr release from prelabeled target cells. The assay system is a "weak" type since specific ^{51}Cr release is low (generally 5-25 percent). Furthermore, we felt that the display of viral antigen at target cell surfaces was not optimal and that spontaneous release of ^{51}Cr from labeled L cells was unsatisfactorily high (30-40 percent) for a "weak" system. Therefore, we began to look for other H-2 compatible target cells and for a gamma-emitting isotope that would have smaller non-specific release.

Lymphoblasts from 2 lines of AKR-derived murine ascites tumors were tested for ^{51}Cr labeling and support of Banzi viral replication. Although both lines labeled satisfactorily, spontaneous release of ^{51}Cr over 16 hours (the incubation period of splenic cells with target cells) was high (average 40-50 percent). More importantly, neither cell type supported viral replication satisfactorily, so further testing was stopped.

We then returned to L-929 cells and found that by increasing the multiplicity of virus inoculated into cultures that the number of infected cells detectable by immunofluorescence 48-72 hours postinfection was 90-95 percent. A review of current literature indicated that ^{75}Se was a useful label for "weak" cytotoxicity systems involving tumor cells as targets. When employed as ^{75}Se-L-methionine, the isotope is adequately bound to tumor cells so that background release is less than 10 percent and radiochemical toxicity to labeled cells is negligible. Preliminary labeling trials of L cells with ^{75}Se-L-methionine had just begun when the contract was terminated.

V. Conclusions

Due to the premature termination of the contract, experiments outlined in the contract proposal were begun, but were not completed with USAMRDC funds.

Work on these experiments has, however, continued with other funds and USAMRDC will receive full acknowledgement as cosponsor of appropriate phases of the research when they are published.

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