ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1989, p. 2126–2131
0066-4804/89/122126-06$02.00/0

Vol. 33, No. 12

Postinfection Therapy of Arbovirus Infections in Mice

I. P. SINGH,[1] D. H. COPPENHAVER,[1] M. SARZOTTI,[1] P. SRIYUKTASUTH,[1] J. POAST,[1] H. B. LEVY,[2] AND S. BARON[1]*

Department of Microbiology, The University of Texas Medical Branch, Galveston, Texas 77550,[1] and National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[2]

Received 10 July 1989/Accepted 30 August 1989

Most antiviral agents are efficacious prophylactically in vivo, and a few are efficacious for postinfection (p.i.) therapy. To explore possibilities for p.i. therapy of encephalogenic Banzi virus (BZV) and Semliki Forest virus infections in mice, we evaluated candidate antiviral therapies after development of the first clinical signs of infection. The earliest clinical indication of BZV viremia in mice is a rise in core body temperature beginning on day 3 p.i. BZV-infected mice showing elevated core body temperatures (≥37.3°C) on days 3 and 4 p.i. were treated intraperitoneally with the interferon inducer poly(ICLC) (80 μg per mouse) and/or specific antiserum. Combined therapy on day 3 of a BZV infection protected over 75% of mice showing clinical evidence of viral disease before treatment. Protection against early brain infection must occur on day 4 p.i., since by that day BZV has started multiplying in the brains of the mice. Significant protection occurred with antiserum alone and increased with poly(ICLC). Similar protection was obtained during Semliki Forest virus viremia, but this infection is so rapid that the first clinical signs are reliably detectable only after viremia.

Although prophylaxis of viral infections may be preferable to therapy, it is often not feasible when vaccines are not available, when vaccines provide only a short duration of immunity, when only a small portion of the population is threatened, or when individuals at risk cannot be identified for chemoprophylaxis. The alternative to prophylaxis is postinfection (p.i.) treatment. However, this treatment is moderately effective at best and often must be given early after infection. Therapy p.i. has been reported for intracerebral infection in mice with encephalomyocarditis virus and arboviruses, including Sindbis virus, Western equine encephalitis virus, and Semliki Forest virus (SFV) (1, 6, 15, 22). Similarly, ribavirin has also been shown recently to be effective in treating hemorrhagic fever with renal syndrome caused by Hantaan virus (7).

Prophylaxis or therapy of most alphavirus and flavivirus infections of humans has not yet been achieved, however, although many of these infections can be serious and life threatening. The present study attempted to achieve p.i. therapy of these infections by starting treatment at the time of viremia, when the virus is spreading to target organs. The stage of viremia was selected for the onset of therapy because it often causes the first identifiable signs and symptoms of disease (e.g., fever or malaise) that would cause a patient to visit a physician for treatment during an epidemic. In the present study, murine models of flavivirus and alphavirus infections were used, and the stage of viremia was identified by detection of virus and the accompanying clinical signs. The results demonstrate successful therapy at the stage of viremia and early clinical signs by combined treatment with high-titered antiserum and an inducer of interferon.

MATERIALS AND METHODS

Mice. Three-week-old female outbred (ICR)Br mice weighing 14 to 15 g were obtained from Harlan Sprague Dawley and maintained in a clean room for a week (according to the "Guide for the Care and Use of Laboratory Animals" [National Institutes of Health publication no. 86-23, revised 1985]). To monitor their state of health, core body temperatures and weights were recorded a day after their arrival and a day before experimentation, by which time the average weight was 18 to 20 g.

Virus. Banzi virus (BZV) SA H336, a flavivirus, and SFV original, an alphavirus, both originally obtained from Robert Shope, Yale University, New Haven, Conn., were passaged intracerebrally in 2- to 3-day-old suckling mice. Stock virus was prepared as a 10% suspension of infected brains in Eagle minimal essential medium (EMEM) with 10% fetal bovine serum. Samples were stored at −70°C. The inoculum for these experiments was prepared by diluting stock virus in EMEM with 2% fetal bovine serum as diluent.

Poly(ICLC). The biological response modifier poly(ICLC) was prepared by the Pharmaceutical Service, College of Pharmacy, University of Iowa, Iowa City. Poly(ICLC) is a stabilized, synthetic, double-stranded polyriboinosinic-polyribocytidilic acid that confers an antiviral state by stimulation of the immune response (3, 21) and induction of interferon production (10, 11). Each milliliter contained 2 mg of poly(ICLC), 1.5 mg of poly-L-lysine, and 5 mg of carboxymethyl cellulose in a 0.9% sodium chloride solution. This mixture was diluted in EMEM, and the pH was adjusted to 7.3.

Immunoglobulin preparation. Immunoglobulins to BZV were prepared in mice according to a standard protocol (16). A 10% mouse brain suspension of virus inactivated with 0.2% β-propiolactone (12) was used as an antigen to immunize mice. The ascitic fluid harvested from hyperimmunized mice was pooled, clarified by centrifugation and treatment with CDR cellulose (Whatman Inc., Clifton, N.J.), and filter sterilized. Samples of ascitic fluid were used in protection and neutralization experiments without further purification.

Antiserum to SFV was raised in rabbits by a procedure designed to eliminate production of anti-mouse and other antibodies not directed against the viral immunogen (R. A. Shope, personal communication). Stock SFV was propagated in rabbit skin fibroblast (RK13) cultures maintained in EMEM supplemented with 10% normal rabbit serum (Sigma Chemical Co., St. Louis, Mo.). SFV antigen (3×10^5

* Corresponding author.

PFU/ml) was prepared by inactivating this virus with 0.2% β-propiolactone at 37°C for 2 h (12) and then homogenizing it with an equal amount of complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). New Zealand White rabbits (2,600 g) were administered the antigen via the subcutaneous route in the neck region; after a week they were injected via the ear vein with two doses of live SFV (2×10^5 PFU) at weekly intervals. One week after the last injection of antigen, rabbits were bled from the ear vein by using a winged infusion set with a 21-gauge needle. To obtain a high-titered antiserum, rabbits were injected intravenously with booster doses of live antigen a week before each subsequent bleeding. The harvested serum was clarified and stored at −20°C until used.

The neutralizing-antibody titer was determined by a plaque neutralization test (4). One neutralizing unit of antibody was defined as the dilution inhibiting 50% of the viral plaques in chick embryonic cell monolayers.

Assay of virus in blood and brain. To correlate clinical indicators with the spread of virus in the body, the virus was assayed in blood and the primary target organ (brain). Thirty to 35 mice were infected with 3 LD_{75}s (75% lethal doses) of the virus. Each day, blood samples and brains from five mice were collected. Heparinized blood samples were obtained by orbital sinus bleeding, diluted 1:5 or 1:10 in EMEM with 2% fetal bovine serum, and centrifuged at 733 × *g* for 10 min. The supernatant was decanted and frozen at −70°C for virus assay. The number of PFU was determined on chick embryonic cell monolayers in microdilution plates. After blood collection, the mice were sacrificed by ether anesthesia and their brains were removed aseptically. The brains of five mice per day were homogenized and suspended in EMEM with 2% fetal bovine serum to obtain a 10% suspension (wt/vol), which was then centrifuged at 733 × *g* for 10 min. The supernatant was decanted, and the suspension was frozen at −70°C for subsequent virus assay. The dilution factors associated with sample collection and virus titrations in these experiments were such that virus levels of less than 280 PFU of serum per ml or less than 280 PFU per brain would not be detected.

In vivo models. Potential clinical indicators of model BZV and SFV murine infections were studied first. The core body temperatures and weights of 10 mice infected intraperitoneally (i.p.) with 3 LD_{75}s of virus and of 10 uninfected mice were recorded from day 0 throughout the course of infection. Temperatures were taken rectally with a Solomat platinum resistance temperature detector electronic thermometer.

For therapeutic intervention, groups of mice were infected with 3 LD_{75}s of BZV or SFV i.p. Since the earliest manifestation of BZV infection was found to be fever on day 3 p.i., mice with fever (≥37.3°C) were separated and distributed equally into four groups. They were treated i.p., starting on days 3 and 4 p.i., with poly(ICLC) (80 μg per mouse) alone, antiserum to BZV (3,900 U per mouse) alone, or a combination of the same doses of poly(ICLC) and antiserum to BZV or were left untreated (virus control). Similarly, SFV-infected mice were treated with an analogous combination therapy regimen on day 2 p.i.; doses of antiserum to SFV were 500 or 1,000 U per mouse. Since no reliable clinical correlates of SFV infection were found, therapy was initiated on day 2 p.i. This was based on experimental evidence indicating that the onset of viremia occurs at day 1 p.i. and on subsequent detection of significant titers of virus in brain tissues on day 2 p.i. Mice were examined twice daily for signs of illness and death until at least 3 days following the last mortality (generally days 15 to 18), during which time no additional mice became ill.

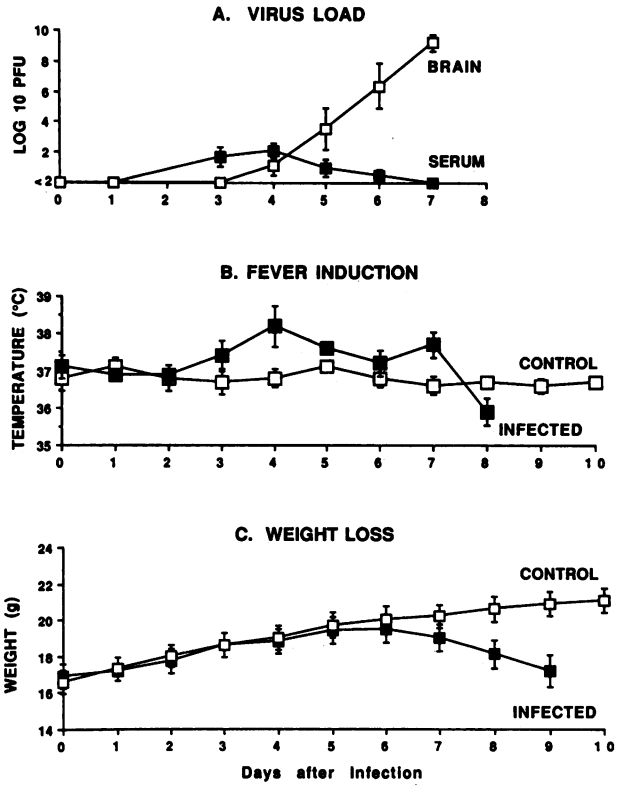

FIG. 1. Clinical indicators of BZV infection in ICR mice. (A) Viremia was determined by individually determining titers of the virus in blood and brains of five mice per day as described in the text. In parallel, core body temperatures (B) and weights (C) of 10 infected (3 LD_{75}s of BZV) and 10 uninfected mice were measured throughout the course of the infection. Standard deviation is shown by the error bars.

Statistical analysis. Weights and temperatures of control and infected mice were analyzed by using Fisher's exact test. Data from drug treatment groups were compared with data from virus control mice by using Fisher's exact test. Combination therapy data were analyzed by using a chi-square test.

RESULTS

Detection of the clinical markers associated with viremia. To determine the first signs of infection that might correlate with viremia, mice were infected with 3 LD_{75}s of BZV or SFV; infection was followed by daily determinations of weight, body temperature, viremia, virus levels in the brain, and changes in motor activity. BZV-infected mice developed viremia over days 3 to 5, which correlates with the first increase in body temperature (Fig. 1A). A rise in core body temperature (≥37.3°C) was consistently detected by day 3 in infected mice (Fig. 1B). This temperature change was significant ($P < 0.01$; Fisher's exact test) and represented the earliest detectable clinical correlate of BZV infection. The first fever response preceded the earliest detection of virus in the brains of infected mice. There was no consistent decrease in normal weight gain in the early viremic phase of infection (Fig. 1C); however, during the course of the subsequent encephalitis, both body temperature and weight

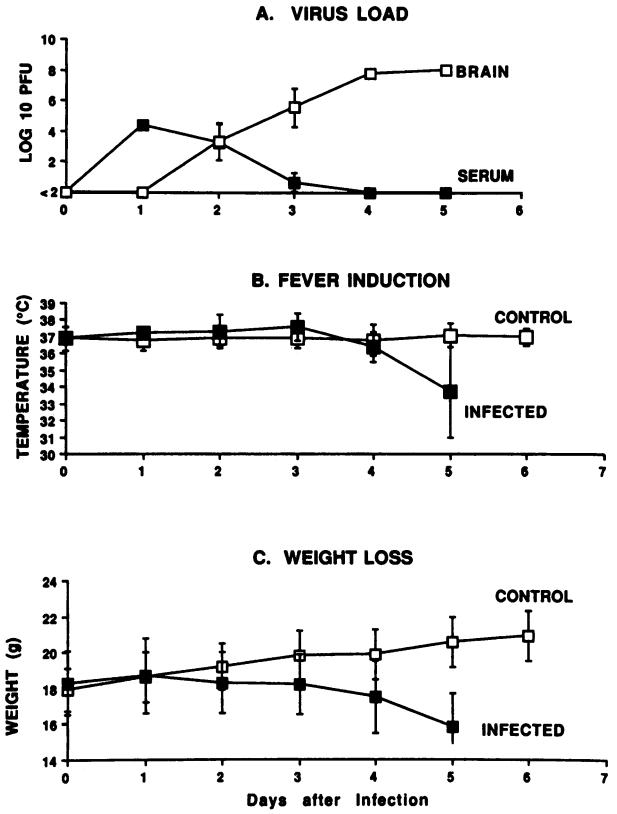

FIG. 2. Clinical indicators of SFV infection in ICR mice. (A) Viremia was determined by individually determining titers of the virus in blood and brains of five mice per day as described in the text. (B) In parallel, core body temperatures of 10 infected (3 LD_{75}s of SFV) and 10 uninfected mice were recorded throughout the course of the infection. (C) Body weights of mice, used for measuring core body temperature mentioned above were recorded. Standard deviation is shown by the error bars.

decreased substantially. Motor activity and behavior also remained normal until the onset of encephalitis (data not shown). These findings indicated that the febrile response was a reliable, uniform correlate of viremia in BZV infection and therefore could serve as a clinical marker for initiation of late therapy in attempts to prevent BZV encephalitis. In the case of the more rapidly progressing SFV infection, viremia was detected on day 1 p.i., and there was no consistent clinical correlate (Fig. 2).

Therapy during viremia. Mice were infected with 3 LD_{75}s of challenge virus i.p. on day 0. For p.i. therapy, only presumptively viremic mice were included. BZV-infected mice with body temperatures of ≥37.3°C on day 3 p.i. were considered viremic and treated as described below. Nonfevered animals were presumed to include those with a slower course of infection and thus were not used in protection experiments. Typically, 60% of BZV-infected mice developed fever by day 3 p.i.

For combined therapy in BZV infections, mice were treated i.p. with 3,900 U of antibody and 80 μg of poly (ICLC) per mouse. SFV-infected mice were treated i.p. with 1,000 or 500 U of antibody and 80 μg of poly(ICLC). In control mice, this dose of poly(ICLC) induced peak mean interferon titers in serum of 88,680 U/ml at 6 h. Such combined therapy protected BZV-infected mice from lethal encephalitis when initiated 72 h p.i. (during the period of peak viremia) (Fig. 3) and at 96 h p.i. (during continued viremia), when the virus had initiated multiplication in the brains of at least 33% of the mice (Fig. 4).

Similar results were achieved with combined therapy at 48 h p.i. (Fig. 5) for presumptively viremic SFV-infected mice. Sixty percent of mice had virus in the brain by 48 h p.i.; greater than 95% of control SFV-infected mice showed detectable viremia by 48 h p.i. (data not shown). No significant protection was recorded with this treatment when intervention was delayed to 72 h p.i., by which point the virus had multiplied to a high titer in the brain.

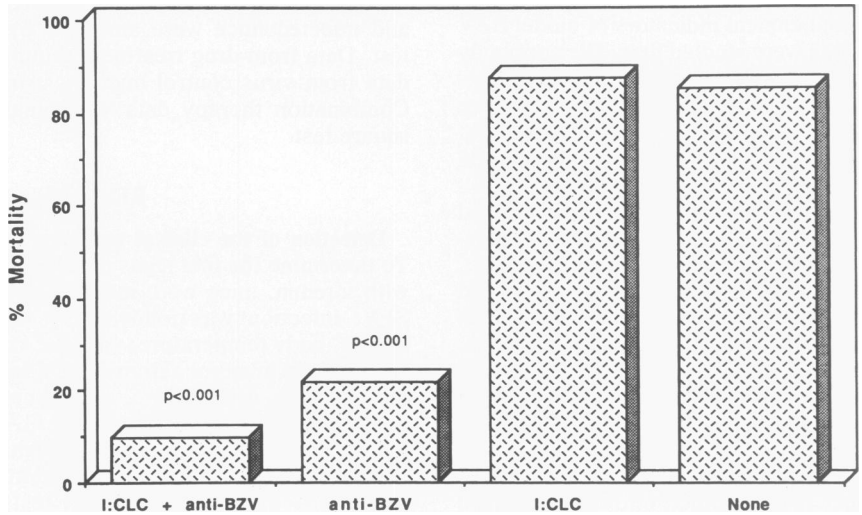

FIG. 3. Combination therapy with poly(ICLC) and homologous antiserum for BZV-infected mice at 72 h p.i. Groups of 40 or 41 mice developing fever (≥37.3°C) on day 3 p.i. were administered either 80 μg of poly(ICLC) per mouse or 3,900 U of antiserum to BZV per mouse or both. Thirty mice served as virus controls. The protection obtained with combined treatment compared with that obtained with virus controls was statistically significant ($P \leq 0.001$) but was not significantly enhanced compared with protection obtained with antiserum alone ($P = 0.08$). Results of Fisher's exact tests for treatment groups compared with untreated controls are shown. The data represent pooled results of four independent series of experiments.

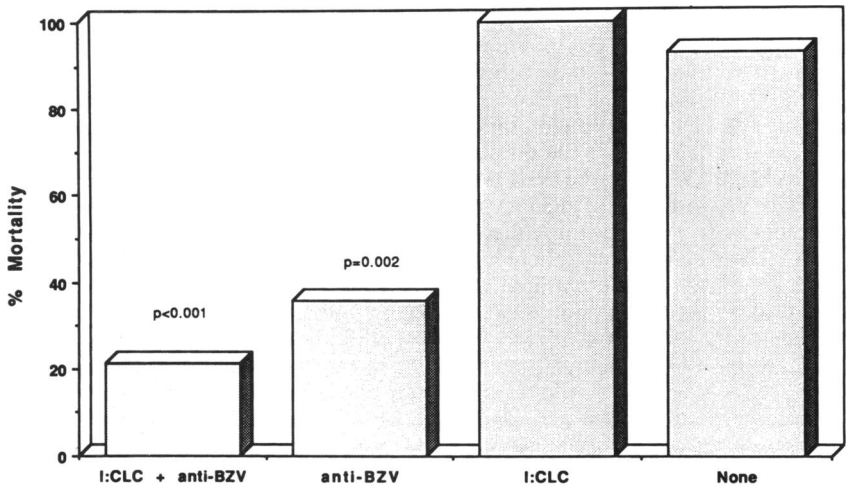

FIG. 4. Combination therapy with poly(ICLC) and homologous antiserum for BZV-infected mice at 96 h p.i. Groups of 14 mice developing fever (≥37.3°C) on day 3 p.i. were given either 80 μg of poly(ICLC) per mouse or 3,900 U of antiserum to BZV per mouse or both. The protection achieved by combined therapy compared with results for virus controls was statistically significant ($P \leq 0.001$; Fisher's exact test) but was not significantly enhanced compared with protection obtained by antiserum treatment ($P = 0.2$).

DISCUSSION

As more potent antiviral therapies become available, prospects for the treatment of viral diseases shortly after the onset of infection improve. In the case of flavivirus and alphavirus infections, the first symptoms that might cause a patient to visit a physician for treatment include malaise, fever, loss of appetite, and loss of body weight, all of which can occur during the viremic phase of the infection when the virus is spreading to the target organs. We have attempted to model such a disease course with BZV and SFV infections of mice. During the viremic phase of BZV infections, we observed a definite and uniform febrile response on day 3 p.i. Using the febrile response as a sign of viremia, we provided therapy to BZV-infected mice using two antiviral agents in combination.

Although the data clearly indicate that there is a tendency for SFV-infected animals to have core body temperatures which are elevated relative to those of controls on days 1 to 3 p.i. and loss of body weight beginning on day 2 p.i., these trends were not statistically reliable markers of the infection (Fig. 2). There is a considerable degree of overlap between control and experimental group ranges for both parameters. This may relate to normal biological variation in an extremely rapidly progressing viral infection. SFV viremia occurs on day 1 p.i. and is followed by detectable virus in the brain on day 2 p.i. Therefore, SFV-infected mice were given

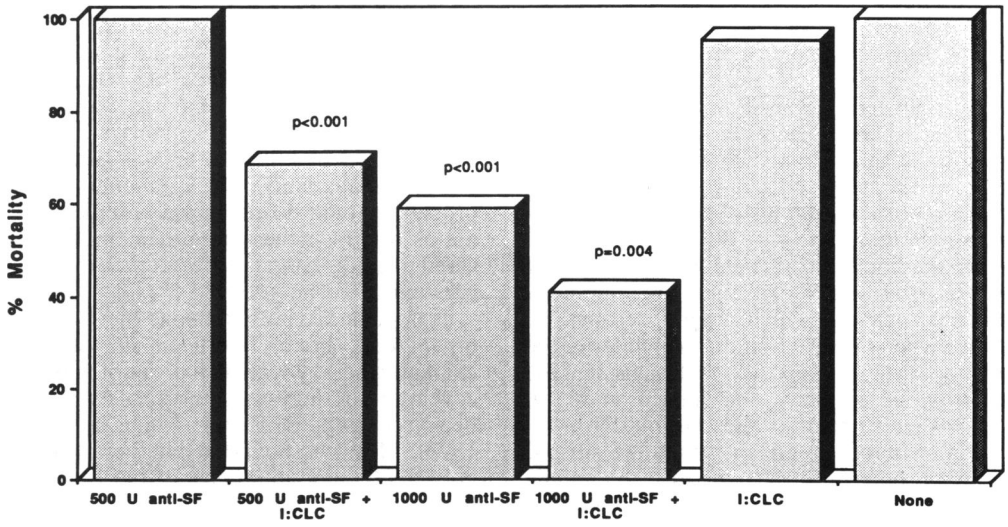

FIG. 5. Combination therapeutic intervention of SFV infection in mice at 48 h p.i., with poly(ICLC) and homologous antiserum. Groups of 22 mice on day 2 p.i. were given 80 μg of poly(ICLC) per mouse or antiserum to SFV (1,000 or 500 U per mouse) or both. The protection achieved by combined therapies of poly(ICLC) (1,000 and 500 U per mouse) compared with results for untreated controls is shown (Fisher's exact test). Combined treatment was significantly more effective than both single-drug controls [$P = 0.004$ versus anti-SFV; $P = 0.02$ versus poly(ICLC)] for the group receiving 500 U of anti-SFV. For the group receiving 1,000 of anti-SFV, the increased protection of the combination treatment group was significantly enhanced only compared with protection of the poly(ICLC) control ($P < 0.001$). The data represent pooled results of two independent experiments.

therapy on day 2 p.i., corresponding to viremia and appearance of virus in the brains of at least 60% of the mice. Intervention at day 2 of an SFV infection is thus roughly equivalent to intervention at day 4 of a BZV infection.

It is possible to rescue a significant portion of virally infected mice when treatment is initiated after the onset of viremia and when the virus has begun to reach the brain (Fig. 3 to 5). Interestingly, although we were able to significantly increase survival by treatment with specific antibodies in combination with poly(ICLC), the mean day of death of mice which did succumb was not significantly different in any of the treatment groups from that of untreated controls (data not shown). It could be argued that this bimodal response (rapid death or survival) means that we were actually saving only those mice which happened to be slowest in developing the disease. Two lines of evidence argue against this conclusion, however. First, in one experiment we treated nonfevered (<37.3°C) mice on day 3 of a BZV infection in addition to the fevered mice. In that experiment, the combined treatment with anti-BZV and poly(ICLC) rescued all of the nonfevered group versus only 72% of the fevered group (data not shown). Although the number of treated nonfevered mice was small, this result is consistent with our successful isolation of the viremic mice for treatment in our BZV experiments. Second, and more dramatic, a number of mice survived throughout these experiments, even though encephalitis progressed to partial paralysis. In none of our experiments with these or other antiviral agents have any untreated control mice survived the onset of encephalitic paralysis. The survival of these partially paralyzed mice after therapeutic intervention with specific antibodies and poly(ICLC) demonstrates that the treatment is capable of halting even highly progressed infections and proves that our results do not merely reflect the survival of those organisms in which intervention was initiated before the progression to significant infection.

In the present study, we examined the possibility of combination treatment with two proven antiviral agents. We initially studied both agents in prophylactic systems, where they are extremely effective antiviral agents even at low doses. For example, doses of poly(ICLC) as low as 0.8 μg per 20-g mouse can fully protect mice from a lethal BZV infection when the drug is given prophylactically at the site of the infection (unpublished observations). The protective effect is rapidly lost when given after virus challenge, however. Intervention at times greater than 24 h after BZV challenge is unsuccessful at rescuing mice from the infection, even when massive doses (80 μg/20-g mouse) of poly(ICLC) are used. Similarly, although as little as 150 neutralizing U of specific antibody is sufficient to provide total protection against a 3-LD_{75} BZV challenge when administered 24 h before virus challenge, much larger doses must be used to protect mice when intervention is begun later in the course of the infection. Previous reports which suggested the synergistic interaction of antibody and interferon (8, 9) encouraged us to evaluate antibody and the interferon inducer poly(ICLC) in combined treatment. When we studied these two agents in a prophylactic system, with suboptimal doses administered 24 h (antibody) and 6 h [poly(ICLC)] before virus challenge, there was evidence of enhanced protection by the combination treatment compared with protection by single-drug control treatments (unpublished observations). The data presented here reinforce this finding and illustrate the increased efficacy of the combination antiviral regimen. In addition, although a single dose of poly(ICLC) alone was ineffective in protecting mice from BZV or SFV lethal encephalitis when administered after peak viremia had developed, combined therapy with antiserum and poly(ICLC) resulted in greater protection than therapy with antiserum alone.

In this model of therapeutic intervention, a maximum tolerated dose (80 μg per mouse) of poly(ICLC) did not significantly affect the course of the infection; neither survival time nor mean time to death was different from control values. In spite of this lack of measurable amelioration of the disease process, the use of this immunomodulator in combination with specific antibodies consistently increased survival in both BZV and SFV infections over that obtained with antibodies alone. Although this increase was slight and generally of borderline significance (Fig. 3 to 5), the fact that it was invariably found in the individual experiments convinces us of its reality. When taken together with the results of experiments in which these agents gave enhanced prophylactic protection against BZV, it appears that poly(ICLC) and specific antibodies give a positive interaction in the treatment of these arbovirus infections. Thus, in these experiments, combination therapy both increased the effectiveness of the treatment and rescued an antiviral agent [poly(ICLC)] that was ineffective as a therapeutic agent.

The positive interaction of these two antiviral agents in the p.i. therapy model is probably explained by their different and complementary mechanisms of action. Poly(ICLC) given alone 48 h after virus infection is not protective via interferon induction or via other immunomodulatory action. However, when the virus load is diminished by specific antibodies given exogenously, the effect of poly(ICLC) becomes relevant. It is unclear at this point whether poly (ICLC) acts via interferon production or immunomodulation in its positive interaction with exogenous antibodies. However, it is clear that the high levels of interferon that are induced by the virus itself are not protective. Thus, we favor the hypothesis that poly(ICLC) interacts with antibodies via its immunomodulatory effect. Obviously, more studies will need to be done to confirm this hypothesis.

The medical significance of these findings is in their implication for the p.i. therapy of human infections in which clinical manifestations occur during viremia. Possible clinical correlates of viremia include fever, malaise, and anorexia. Viremia in humans may occur with infections by flavivirus, alphavirus, arenavirus, varicella virus, picornaviruses, measles virus, and rubella virus. Although the ordinarily moderate clinical manifestations of viremia alone would not invariably cause a patient to visit a physician, they probably would do so during an epidemic. Additional scenarios in which these treatment modalities might be indicated include cases of accidental exposure of laboratory personnel to arboviruses.

Treatment of viral infections that have reached the target organ (e.g., brain) has been shown to be feasible. For example, therapy with high doses of adenine arabinoside and acyclovir for herpes simplex encephalitis in humans has been reported to decrease mortality and morbidity (14, 18–20). Here we show also that therapeutic intervention with poly(ICLC) and antibodies of the early phase of BZV and SFV brain infection protects a significant fraction of mice (Fig. 3 to 5, respectively). At 4 days p.i., BZV is found at a low titer in the brains of at least 33% of infected mice (log 2.5 PFU), and by day 7 it is present in a high titer (log 9.2 PFU) in the brains of all mice tested. The course of SFV infection is much faster than that of BZV. It is first detectable in the brain on day 2 p.i. in 60% of mice (log 3.3 PFU), and by day 4 the virus is present in a high titer (log 7.8 PFU) in all mice.

Because of the dilution factors referred to earlier (see Materials and Methods), these are conservative estimates of the time of appearance of virus in the blood and brain.

These principles of p.i. therapy seem to apply to humans. In a patient with hypoglobulinemia, therapy with sufficient quantities of specific antibodies was first found to be effective in controlling chronic echovirus type 5 encephalitis (17). This use of passive antibody therapy has been used successfully by others for hypogammaglobulinemia with chronic viral infections when the dose of specific antibody was adequate (2, 5, 13).

Taken together, the evidence supports the interpretation that effective therapy may be possible and practical during the viremic phase and early encephalitis of some viral infections.

ACKNOWLEDGMENT

This work was supported by the U.S. Army Medical Research and Development Command under contract no. DAMD 17-86-C-6119.

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