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Impact of Dosing Schedule upon Suppression of a Retrovirus in a Murine Model of AIDS Encephalopathy

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We studied the impact of zidovudine (AZT) in Cas-Br-M murine leukemia virus-infected NFS-N mice after administration by once-daily bolus or continuous infusion. While higher peak concentrations of AZT were achieved by once-daily dosing, continuous AZT infusion at 25 μg/h maintained levels >1 μM in plasma and >0.2 μM in the brain. Continuous infusion provided significantly better viral inhibition, even though total doses were only one-third that of the once-daily therapy group.

Optimization of antiviral chemotherapy is more complex than that seen with traditional antibacterial chemotherapy, particularly when nucleoside analogs are being employed. This is because the active species is not the parent nucleoside but rather is a metabolite of the parent compound, usually the triphosphate (7). Consequently, the concentration-time curve in plasma may be less directly linked to the therapeutic effects of the drug. Little information is available regarding the impact of the administration schedule on the antiviral activities of different antiretroviral compounds.

Central nervous system (CNS) infection with the human immunodeficiency virus has recently been found to cause a great deal of morbidity (3, 4, 11, 13). Human immunodeficiency virus type 1 replicates in the CNS, and high levels of virus expression have been correlated with encephalopathy and myelopathy (8, 10, 17). We felt that it was important to examine the penetration of zidovudine (AZT) into this reservoir of the virus and to specifically examine the impact of the dosing schedule on suppression of a murine retrovirus replicating at this site.

Mice. Pregnant NFS/N mice were obtained from the Animal Program administered by the Animal Genetics and Production Branch of the National Cancer Institute. To minimize exogenous infection, animals were housed singly in microisolator cages for filtration of room air. Sentinel mice were routinely monitored for infection by exogenous viruses in the environment, including mouse hepatitis virus, pneumonia virus of mice, minute virus of mice, reovirus type 3, lymphocytic choriomeningitis virus, and Sendai virus.

Inoculation and clinical observation. Pregnant NFS/N mice were observed daily, and their pups were inoculated with Cas-Br-M murine leukemia virus (MuLV [10^5 PFU/ml, 0.03 ml intracranially]) 1 to 2 days after birth. Pups were weaned at 21 to 28 days of age and housed by sex, litter, and treatment. Observations of general health status were performed daily.

AZT administration. Four groups of mice (11 per group) were evaluated. The groups were (i) uninfected and untreated, (ii) infected and untreated, (iii) infected and treated with 100 mg of AZT per kg of body weight intraperitoneally once daily, and (iv) virus infected and treated with ~30 mg of AZT per kg of body weight administered subcutaneously as a continuous rate infusion. For constant infusion administration, ALZET 2001 osmotic minipumps (ALZET Corporation, Palo Alto, Calif.) were loaded with 200 μl of 25 mg of AZT per ml in saline (the limit of solubility of AZT). The sterile osmotic minipumps were implanted subcutaneously in the scapular region under a short-acting general anesthetic. ALZET 2001 pumps deliver 1 μl/h (25 μg/h or 24 to 30 mg/kg/day for 20- to 25-g mice) for 7 days.

HPLC assay of AZT concentrations. The high-performance liquid chromatography (HPLC) method used to determine the concentration of AZT in plasma has been described previously (1). By this method, AZT concentrations were linear from 0.1 μM (27 ng/ml, the limit of detection) to 200 μM, with a coefficient of variation of <10% between assays over 2 years. Brain homogenates were assayed against a standard curve derived from the homogenized brains of infected untreated mice which had been spiked with AZT and internal standard.

Preparation of tissue homogenates and virus titration. Mice were euthanized and bled by cardiac puncture. The brain was removed and rapidly frozen in methanol-dry ice or liquid nitrogen and stored at −80°C or below. The protein content of the homogenates in phosphate-buffered saline was determined by Coomassie blue dye binding (Bio-Rad, Rockville Center, N.Y.). The titer of Cas-Br-M MuLV in the brain was determined with an XC plaque assay after infection of SC-1 fibroblasts in duplicate with serial dilutions (10-fold) of tissue homogenate in Eagle's minimal essential tissue culture medium without serum (14). The MuLV titer is expressed as XC PFU/0.1 g of brain tissue. The AZT 50% inhibitory concentration was 0.02 μM.

Polyacrylamide gel electrophoresis and immunoblotting. Samples (3 to 400 ng of protein) were analyzed on 3-mm-thick, 16-cm-long sodium dodecyl sulfate–12% polyacrylamide slab gels (2). Transfer of proteins to nitrocellulose was for 16 h at 19 V with Tris-glycine-methanol buffer as described by Burnette (5). Viral proteins were identified after reaction with primary antisera (goat anti-Rauscher MuLV p30 and ^{125}I-labeled protein G (Amersham, Oak Park, Ill.). Autoradiography was performed with Chronex 4 film and Quanta III enhancing screens at −70°C.

Statistical analysis. The means and the standard deviations of all treatment groups were calculated, and the values were compared by using one-way analysis of variance between treatments. The level of significance was set at $P \leq 0.05$ and

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maintained in the multipairwise comparisons according to the method of Newman and Keuls (16).

Pharmacokinetic analysis. The area under the concentration-time curve in plasma was determined by the LAGRAN program of Rocci, Jr., and Jusko (12). Terminal rate constants were determined by weighted nonlinear least-squares regression employing the ADAPT II package of programs of D'Argenio (6). In general, weighting was by the inverse of the assay variance. Model discrimination for terminal rate constant estimation was by the Akaike information criterion (18). Penetration ratios were determined as the ratio of the area under the concentration-time curves in the brain and the plasma for the intermittent therapy group and by the ratio of mean steady-state concentration in the brain and plasma for the continuous infusion group.

Therapy with AZT was initiated 3 weeks after the intracerebral inoculation of the virus, because preliminary studies showed that this was the earliest time that Cas-Br-M virus would be reproducibly identified as actively replicating in the CNS.

Concentrations of AZT in plasma achieved in infected mice by 100-mg/kg intermittent intraperitoneal injection are displayed in Fig. 1A. A single injection of a large (100-mg/kg) dose of AZT produced transient peaks of AZT in excess of 300 μM in the plasma of the infected male and female NFS/N mice. The terminal half-life of AZT is less than 20 min in NFS/N mice, which results in an exposure profile in which AZT is not present for >75% of the dosing interval. Continuous infusion produces steady concentrations of 1 to 2 μM AZT in plasma. Concentrations of AZT in brain homogenates were 0.1 to 0.3 μM for the continuous infusion group. In Fig. 1B, we show the AZT concentrations in plasma and in homogenates from the brain and spleen after administration of a 50-mg/kg dose. Levels of AZT achieved within the CNS are 1/10th to 1/30th those found in plasma and spleen tissue, respectively. Peak concentrations of AZT in the brain with the 50-mg/kg dose are more than 20-fold higher than the levels produced by the continuous infusion regimen. However, there is a very short terminal half-life, and concentrations in the brain drop below the sensitivity of the assay by 4 h postadministration. This is indicative that AZT is not present for >75% of the dosing interval. In contrast, ALZET minipump-mediated infusion maintained steady-state concentrations in brain homogenates of 0.2 to 0.3 μM over the entire treatment period. As expected, the continuous infusion and intermittent administration regimens produced penetration ratios (area under the concentration-time curve in the brain/area under the concentration-time curve in plasma ratio) which were similar, at 22 and 14% for the continuous and intermittent dosing groups, respectively.

Untreated virus-infected animals had a mean (± standard deviation) of 39.2 ± 29.9 XC PFU/0.1 g of brain homogenates. The 100-mg/kg once-daily intraperitoneally dosed group had 37.4 ± 18.0 XC PFU/0.1 g of brain homogenate, which was not statistically different from that of the control group. Animals continuously infused with AZT had an average of 15.4 ± 16.7 XC PFU/0.1 g of brain tissue assayed. This was statistically different from either of the other two groups (P = 0.03). Virological findings were confirmed by analysis of viral protein p30 in the CNS. Densitometric measurements of immunoblots of brain homogenate from untreated mice showed levels of p30 threefold higher than those of mice treated with AZT by continuous infusion (Fig. 2).

Since nucleoside analogs must penetrate cells and be phosphorylated to the triphosphate, the concentration of the active moiety at the infection site should correlate with the viral suppression which is seen.

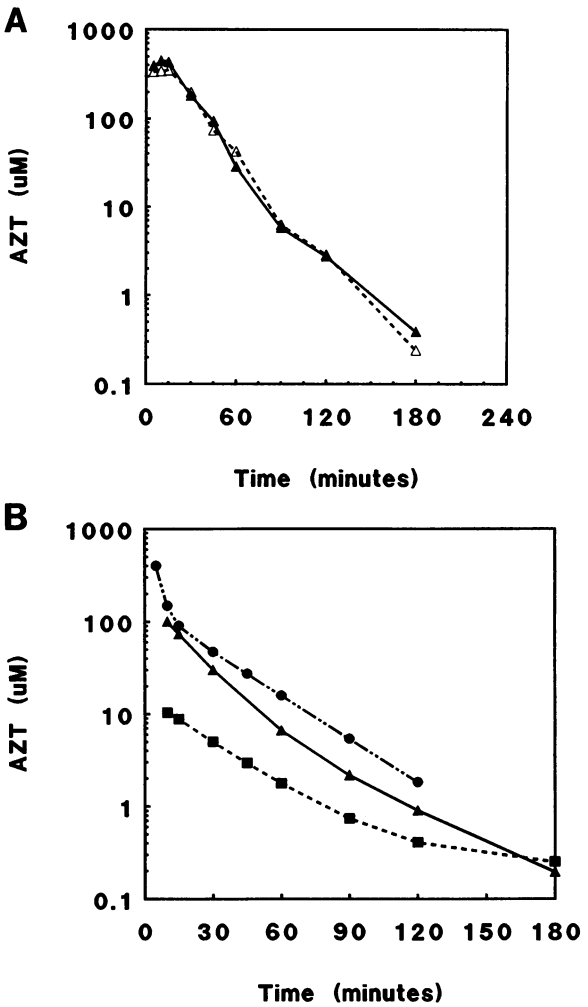

FIG. 1. (A) Concentrations of AZT in plasma reached after injection of 100 mg/kg as a single intraperitoneal bolus. Blood was obtained by cardiac puncture of male (△) and female (▲) mice at the indicated time points. The concentration of AZT in plasma recovered after centrifugation was measured by HPLC as described in Materials and Methods. (B) Pharmacokinetics of AZT in plasma (▲), the spleen (●), and the brain (■) after administration of 50 mg/kg as a single intravenous bolus. Mice were euthanized at the indicated time points after intravenous administration of 50 mg of AZT per kg, and blood, the brain, and the spleen were removed. Plasma and tissue homogenates were prepared, and the concentration of AZT was determined as described in Materials and Methods.

It was the aim of this study to examine viral suppression at extremes of administration schedule, comparing large doses given once daily to continuous infusion of the drug. If the peak concentration or area under the concentration-time curve was linked to outcome, once-daily administration would be superior or equivalent. If the time that the drug spent above an inhibitory threshold was more important, continuous infusion administration would be superior.

In our experiments, increased efficacy of continuous infusion of AZT was found even though total doses of intermittently administered AZT exceeded that of continuous infusion by more than threefold, indicating that the time concentrations remain above an inhibitory threshold is the dynamic variable

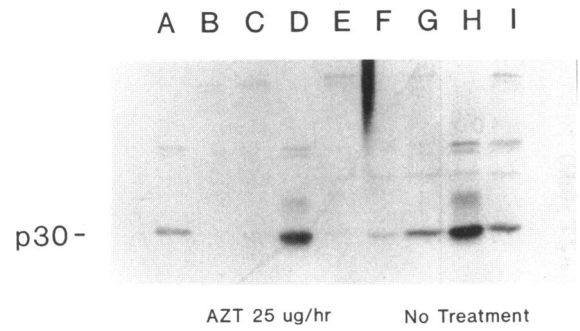

FIG. 2. Analysis of MuLV p30 protein in the spleen and CNS tissues of NFS/N mice treated with continuous infusion of AZT. Two mice treated with AZT and their untreated counterparts were sacrificed for analysis of virus-related protein in the CNS. Their brains were bisected into the cerebrum and cerebellar sections prior to preparation of the homogenate. Spleens and spinal cords removed from the same animals were included as a comparison. After polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose and analyzed as described in Materials and Methods. Lanes: A, purified MuLV; B, C, D, and E, cerebrum, cerebellum, spleen, and spinal cord, respectively, from mice continuously infused with 25 μg of AZT per h; F, G, H, and I, cerebrum, cerebellum, spleen, and spinal cord, respectively, from untreated mice.

most closely linked to viral suppression. Likewise, Sinet et al. (15) recently reported that continuous infusion of AZT consistently inhibited Friend virus-induced splenomegaly more effectively than intermittent dosing.

The explanation for these findings is unclear. However, we speculate that the reaction which takes AZT from the parent compound to the monophosphate is Michaelis-Menten in nature. Large intermittent doses of AZT may produce concentrations of drug which greatly exceed the K_m of this thymidine kinase. Therefore, despite the high concentration of the parent compound, only a small increment in AZT monophosphate is generated during the time when high concentrations of AZT are present. With the rapid half-life of the drug in mice, a steep concentration gradient of drug movement from inside the cell to outside the cell may develop. The uncharged parent drug can then rapidly efflux, leaving only minimal amounts of drug available for further phosphorylation. Finally, the extended dosing interval allows for long periods of time with very low concentrations of AZT triphosphate.

In contrast, even though only low concentrations of drug were present in brain in the continuous infusion group (0.2 to 0.3 μM), they were there continuously. Phosphorylated molecules have an alteration in their elimination half-life from cells because of ion trapping, possibly leading to some accumulation of drug intracellularly and the ultimate production of more triphosphorylated species than were present with the intermittent regimen. Obviously, these explanations need to be demonstrated experimentally.

These data do not necessarily support continuous infusion as the administration mode of choice for human immunodeficiency virus-infected patients. The phosphorylation patterns of humans are quite different from those seen in murine species. In humans there is a mono- to diphosphate block. Also, as demonstrated by Ho and Hitchcock (9), removal of external drug from the in vitro culture system results in a rapid decline in the amount of monophosphate in the cells, declining from circa 20,000 cpm to circa 5,000 cpm over 2 h. Currently, an administration schedule of 300 mg of AZT every 12 h is being compared with a schedule of 100 mg every 4 h in a randomized clinical trial. While the outcome of this clinical trial is not yet known, it is likely that the group with the long dosing interval will have prolonged periods (6 to 8 h) of very low concentrations of the parent compound in plasma. Whether this will result in a significantly worse clinical outcome will probably be determined by the fall in the amount of intracellular monophosphate. If this amount does not fall below the K_m of the thymidylate kinase, the throughput of drug to the triphosphate will not be adversely affected and the outcomes will be the same. If, alternatively, the intracellular monophosphate concentration falls significantly below the K_m, this decline, coupled with low parent compound concentrations for a great percentage of the dosing interval, will likely result in decreased concentrations of triphosphate and a poorer clinical response.

The schedule of administration may have a significant impact on the therapeutic efficacy of antiretroviral agents, as demonstrated here. Furthermore, we speculate that the determination of the kinetic parameters for each reaction which leads to production of the active inhibitory moiety is of utmost importance in determining the proper mode of administration of nucleoside analogs and perhaps other retroviral agents which undergo metabolic activation.

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