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Effect of 3′azidothymidine administered in drinking water or by continuous infusion on the development of MAIDS

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Summary

LP-BM5 MuLV infection of C57BL/6 mice induces a well characterized, lymphoproliferative, immunodeficiency disease (MAIDS), which is useful for evaluation of potential antiviral agents, because of the reproducibility of virological and clinical endpoints. This MAIDS retrovirus model was used to evaluate 3′azido-2,3′dideoxythymidine (AZT), using different doses, methods of administration and timing for initiation and continuation of therapy. AZT therapy 1 mg/ml in the drinking water given 30 days prior to virus challenge, and continued for 16 weeks, prevented LP-BM5 MuLV dissemination and disease in 13 of 15 treated mice. Efficacy was dose dependent for AZT concentrations of 1, 0.5, and 0.1 mg/ml in drinking water. One mg/ml AZT was most effective in preventing infection if therapy was begun within days prior to virus challenge or within the first four hours after virus inoculation. If treatment was initiated later, disease was delayed. Continuous infusion of AZT, 25 μg/h, was effective since virus was not detected in spleens of any mice during the 21 days of AZT treatment. However, after treatment was stopped treated mice became virus positive and disease progressed. Likewise, AZT administration at 1 mg/ml in the drinking water for only 21 days post virus inoculation (p.i.), was not sufficient to prevent virus dissemination or disease.

Azidothymidine; Zidovudine; MAIDS; Antiviral therapy; LP-BM5; MuLV

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Introduction

Several murine model systems are available to test the in vivo efficacy of anti-retroviral agents, such as 3′azido-3′deoxythymidine (AZT), which affect steps in virus replication common to retroviruses. These include the SCID mouse reconstituted with human cells and HIV (McCune et al., 1988,1990; Namikawa et al., 1988; Mosier et al., 1988), the Rauscher MuLV model (Ruprecht et al., 1987), the Cas-Br-M or Cas-Br-E MuLV model (Sharpe et al., 1987; Bilello et al., 1990), the Friend MuLV (Sidwell et al., 1966; Morrey et al., 1990) and the LP-BM5 MuLV model (Mosier et al., 1985, 1986). While HIV infection in the SCID mouse reconstituted with human cells appears to be the most relevant to AIDS, the limitations of this SCID model include biohazards associated with HIV infection, the use of human fetal tissue in some systems, lack of clinical endpoints and possible phenotypic mixing with endogenous murine retroviruses (Lusso et al., 1990). In all these murine models of retrovirus infection, AZT could be administered at doses which prevented infection.

LP-BM5 MuLV-induced lymphoproliferative immunodeficiency disease (MAIDS) is well characterized and reproducible in the laboratory (Mosier et al., 1985, 1986; Yetter et al., 1986; Klinken et al., 1988). The progression of MAIDS has been defined for various inbred mouse strains (Hartley et al., 1989). LP-BM5 MuLV infection of susceptible mice has been used as a model system to determine the efficacy of AZT in vivo. Several laboratories have confirmed the efficacy of oral AZT treatment although inhibition of LP-BM5 replication and disease progression depended upon when therapy was initiated and the dose administered. Gangemi et al. (1989) reported that oral AZT therapy (0.2 mg/ml in the drinking water) from 0 to 22 days p.i. did not prevent disease but was effective in reducing virus titer and the extent of immunosuppression. No reduction in virus titer or delay in disease progression were observed when AZT was administered to C57BL/6 mice beginning 23 days p.i. Basham et al. (1990) demonstrated continuous low dose (0.1 and 0.25 mg/ml) AZT reduced ecotropic and mink cell focus forming virus titers and prolonged survival of LP-BM5 MuLV infected mice. Oral AZT treatment (0.25 mg/ml) also delayed the early LP-BM5-induced alterations in lymphoid cell populations (Portnoi et al., 1990). Only one study reported prevention of infection with LP-BM5 MuLV (Ohnota et al., 1990). They reported that continuous oral AZT treatment (1 mg/ml) prevented LP-BM5-induced immunodeficiency in C57BL/10 mice if treatment was initiated at the time of infection, but not if treatment was delayed until 1 week post virus exposure.

The objective of this study was to use the MAIDS model to optimize therapeutic intervention with AZT by defining the optimum dose and route of administration.

Materials and Methods

Mice

Virus-free adult C57BL/6 mice were obtained from the Animal Program administered by the Animal Genetics and Production Branch of the National Cancer Institute, and were allowed to acclimate to the facilities before studies were initiated. To minimize exogenous infection mice were maintained in microisolator cages and handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH No. 85-23, 1985). Cages, bedding and food were autoclaved prior to use and all cage changes and animal handling or monitoring were performed in laminar air flow hoods. Ventilation and air flow in the Animal Facility is set to 12 changes/h. Room temperatures are regulated at 72 $\pm$ 2°F and the rooms are on automatic 12 h light/dark cycles. Mice received Purina Autoclavable Chow and water ad libitum until initiation of the study. Thereafter, water or AZT-containing water was supplied to the mice and water consumption was monitored. Mice were inoculated intraperitoneally with either 0.1 ml of a virus pool or tissue culture media. To assure the microbial integrity of the study, sentinel mice were tested for possible exogenous viral infections including mouse hepatitis, PBM, MVM, reovirus type 3, LCM, and Sendai virus using a M.A.P. test (Litton Bionetics, Charleston, SC). Positive animals were not detected.

Virus preparation

LP-BM5 MuLV used for these studies was prepared from chronically infected SC-1 cells (as previously described by Yetter et al., 1988). Titers of ecotropic MuLV were determined by the XC plaque test (Rowe et al., 1970). Most of the experiments described here were performed using a single pool of LP-BM5 MuLV (pool 59). One tenth ml of this virus pool has been shown to induce MAIDS in 100% of inoculated C57BL/6 mice and contains $\log_{10}$ 5.78 pfu/ml.

Clinical observations and measures of disease progression

Mortality and morbidity as well as general health status (appearance, behavior, appetite and waste elimination) were observed daily. Body weights were recorded weekly. Mice were monitored for LP-BM5 MuLV-induced disease by observation and palpation of the peripheral lymph nodes and spleen. Mice are scored for disease based upon palpable alterations in the size of lymph nodes and spleen. Disease scores 0 through +4 are recorded based upon the following criteria: 0, normal; +1, any detectable increase in the size of a single lymph node; +2, readily detectable greater than 0.3 cm in diameter increase in more than 1 lymph node, and in particular, enlargement of subcervical, brachial or axillary nodes; +3, increased size of all palpable nodes and

palpable splenomegaly ($\geqslant$ 0.3 grams); +4, advanced disease with extensive splenomegaly ($>$ 1.0 grams) and lymphadenopathy. Moribund animals were killed by CO_2 anesthesia and necropsied. Spleen weight and hematocrit were recorded at sacrifice and all major organs were examined grossly. Hematoxylin-eosin stained, formalin-fixed samples of spleen, lymph nodes, thymus, lungs, liver, brain, G.I. tract, bladder, heart, reproductive tissue and kidneys were evaluated histologically. A staging system developed to assess the progression of MAIDS was modified to add the definition of an early reactive phase, an early prodromal phase, and three stages of disease. Disease stage was based on the histopathologic lesions detected in tissue sections. The three levels of the reactive stage were assigned scores of 0.25, 0.50 and 0.75 according to the follicular size, cell type and mitotic activity. The earliest stage at which prodromal MAIDS could be reliably distinguished from reactive stages was given a disease stage score of 1.0. The previously described MAIDS stages 1, 2 and 3 (Hartley et al., 1989) were assigned disease stage scores of 2, 3 and 4 respectively.

AZT

AZT was administered either by continuous infusion or intermittently in the drinking water. For drinking water studies, AZT was dissolved in sterile water at the desired concentrations: 1 mg/ml, 0.5 mg/ml or 0.1 mg/ml and filtered through a 0.45 μm filter. For precise timing of administration at -4 h and $+4$ h post virus challenge, a single loading dose of 0.25 ml of 1.0 mg/ml AZT in drinking water was administered by oral gavage. Water consumption was monitored and samples of water were sequentially analyzed throughout the study by HPLC. The AZT concentrations of 1 mg/ml solutions were 1.042 $\pm$ 0.067, 0.5 mg/ml solutions were 0.490 $\pm$ 0.026, and 0.1 mg/ml solutions were 0.098 $\pm$ 0.007 mg/ml. Water bottles were drained and filled every three days and AZT concentrations were identical for the initial stock solution and water remaining in the water bottle at three days. For constant infusion administration, ALZET 2001 miniosmotic pumps (Alza Corporation, Palo Alto, CA) were loaded with 200 μl of 25 mg/ml AZT in saline (the limit of solubility of AZT). AZT was shown to be stable at body temperature for a minimum of 8 days. The sterile miniosmotic pumps were implanted subcutaneously in the scapular region under short-acting general anesthetic. ALZET 2001 pumps deliver 1 μl/h for 7 days. In order to maintain constant infusion over 21 days, pumps were replaced at the end of the first and second weeks. Pumps delivered 0.6 mg of AZT per day, as compared to 5, 2.5 and 0.4 mg per day for the 1.0, 0.5 and 0.1 mg AZT/ml drinking water groups respectively. No changes in hematocrit, body weight or general health could be attributed to implantation of saline containing miniosmotic pumps in control or infected mice.

HPLC assay of AZT levels

The high performance liquid chromatographic method used to determine the concentration of AZT in plasma will be detailed elsewhere. Briefly, an internal standard (β-AZT) was added to 200–500 μl aliquots of heparinized plasma. AZT in plasma was extracted using a solid phase C18 extraction cartridge (Speed, Applied Separations, Bethlehem, PA) and eluted with methanol. Methanol extracts were evaporated under nitrogen and suspended in 10 mM ammonium acetate. The samples were separated using a Hewlett Packard 1090M HPLC equipped with a Diode Array Detector (absorption wavelength, 267 nm), Brownlee Spheri-5 C-18 reverse phase column (220 × 4.6 mm, Applied Biosystems, Inc., San Jose, CA) using a gradient of 17–30% acetonitrile in 10 mM ammonium acetate. Using this method, AZT concentrations were linear from 0.1 μM (27 ng/ml, limit of detection) to 200 μM with a coefficient of variability of less than 10% between assays over 2 years.

Serum immunoglobulin determination

Blood was obtained by cardiac puncture using a heparinized syringe and transferred to a heparinized hematocrit tube. After centrifugation, hematocrit was recorded and the plasma was removed from the tube and either directly assayed or stored at −70°C. Serum IgM assays were carried out using a competition ELISA modified from one previously described by Yetter et al. (1988). Briefly, polyvinylchloride microtiter ELISA tray wells (Costar, Cambridge, MA) were coated with 100 μl of a purified goat anti-mouse IgM diluted 1:500 in phosphate buffered saline (PBS). The plates were blocked by the addition of 1% BSA in PBS. A standard dilution series, in PBS with 0.05% BSA, was prepared using purified IgM from a murine hybridoma (TEPC-183, Zymed Laboratories, South San Francisco, CA). Mouse serum samples were diluted serially in PBS with 0.05% BSA. Of each dilution 100 μl was added to each well of the anti-IgM coated plate and the plates were incubated at 22°C for 1 h.

Goat anti-mouse IgM labeled with horseradish peroxidase was diluted to 1 μg/ml in 1% BSA PBS. After washing the plates three times 100 μl of anti-IgM was added to each well. Plates were incubated at 22°C for 1 h prior to extensive (6×) washing with 0.05% BSA PBS. To each well 100 μl OPD (*o*-phenylenediamine) solution was added and the reaction stopped with 2 N H_2SO_4 after 2.5 min of color development. The optical density of the wells was read at 450 nm.

Virus assay

Mice were assayed for the production of ecotropic virus associated with the LP-BM5 MuLV by the infectious center method which has been detailed elsewhere (Hartley and Rowe, 1975). Briefly, washed splenocytes were

suspended in PBS, treated with mitomycin C and plated onto SC-1 cells. When the SC-1 cells grew to confluence they were UV-irradiated and overlaid with XC cells. After three days, the XC cells were fixed and stained and plaques counted.

Statistical analysis

The mean and the standard deviation of all treatment groups were calculated and the values were compared using one way Analysis of Variance (ANOVA) between treatments. The level of significance was set at $P \leqslant 0.05$ and maintained in the multipairwise comparisons according to the method of Newman and Keuls (Snedecor and Cochran, 1980)

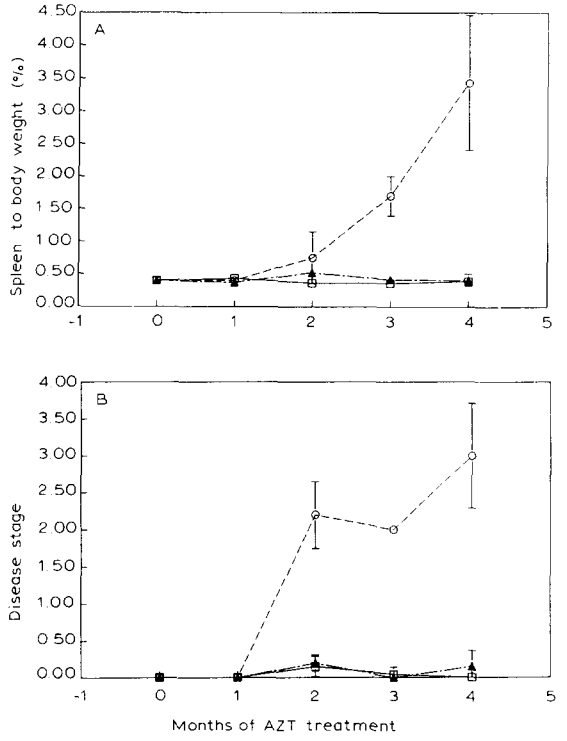

Fig. 1. Spleen to body weight ratios and disease stage score. Groups of 5 mice were killed each month. (A) Body weights were measured prior to euthanasia and spleens were weighed immediately after removal. The spleen to body weight ratio is presented as a percentage and each value is the mean ± standard deviation. (B) Tissues from mice euthanized at monthly intervals were fixed and scored as described. Results are the mean ± standard deviation of 5 animals per group; (□—□), control, uninfected mice; (○—○), LP-BM5 MuLV challenged mice; (▲—▲), AZT treated, LP-BM5 MuLV challenged mice.

Results

When AZT was administered to mice at 1 mg/ml in the drinking water (water consumption: 4.5 ± 0.99 ml/mouse/day), beginning one month prior to challenge with LP-BM5 MuLV and continued for 3 months, disease was prevented. Body weights of LP-BM5 MuLV infected mice receiving AZT (AZT-treated) were not different from sham infected, untreated animals (control) and were significantly lower than untreated LP-BM5 MuLV inoculated mice by 6 weeks p.i. Body weights for control, AZT-treated and LP-BM5 MuLV inoculated mice were respectively 23.8 ± 1.02, 23.8 ± 1.22 and 24.5 ± 1.29 g at 1 month p.i.; 25.8 ± 1.26, 25.1 ± 1.60, and 27.3 ± 1.57 g at 2 months p.i.; and 27.0 ± 1.69, 27.0 ± 1.77, and 29.3 ± 1.58 at three months p.i. The difference in weight was directly related to the degree of lymphadenopathy and splenomegaly in the MAIDS mice. Lymphadenopathy was apparent in the untreated infected mice by 3 weeks p.i., and the spleen/body weight ratios from mice killed at monthly intervals confirm the assessment of lymphadenopathy and splenomegaly in vivo by palpation and gross observations (Fig. 1). AZT-treated, LP-BM5-infected mice did not have increased spleen to body weight ratios. Histopathological examination of selected target tissues from AZT-treated, LP-BM5 MuLV-infected mice also documented the absence of virus associated lesions. Elevations in serum IgM levels associated with the LP-BM5-induced polyclonal B cell proliferation (Mosier et al., 1985; Klinman et al., 1988), were not observed in the control and AZT-treated mice (Fig. 2).

Infectious center assays were performed using splenocytes from the animals whose spleen to body weight ratios and disease stage are presented in Fig. 1. The results indicated that the 1 mg AZT/ml treatment regimen, begun one

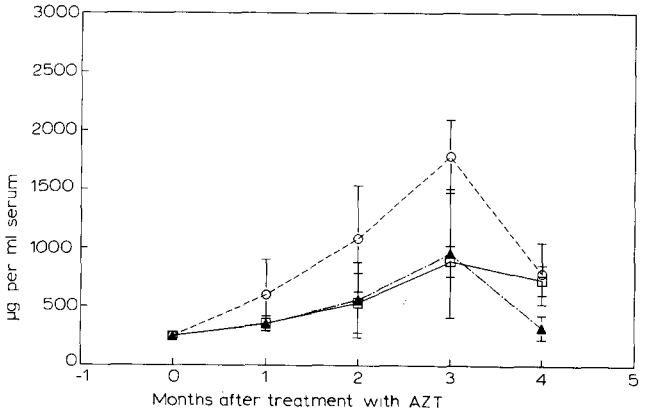

Fig. 2. Serum immunoglobulin levels. Groups of 5 mice were killed each month and blood was collected by cardiac puncture. Serum was prepared and assayed for IgM as described. Results are the mean ± standard deviation of 5 animals per group; (□—□), control, uninfected mice; (○—○), LP-BM5 MuLV challenged mice; (▲—▲), AZT treated, LP-BM5 MuLV challenged mice.

month prior to virus challenge, was able to prevent the dissemination of ecotropic virus. Untreated LP-BM5 MuLV inoculated mice had a mean of 3584 $\pm$ 5888 virus-producing cells/10^6 splenocytes, one month p.i., while virus was not detectable in the splenocytes of control or AZT-treated infected mice. At two and three months p.i., ecotropic virus expression was greater than 10^3 virus-producing cells per 10^6 splenocytes in the 10 of 10 untreated infected mice. Ecotropic virus expression was detected in splenocytes from only 2 of 10 AZT treated infected mice, but the titers were 1000-fold lower than those in the splenocytes from the untreated infected mice. The mean survival of the untreated infected mice was 19 weeks post inoculation while the AZT-treated mice were asymptomatic at 8 months.

In this study, the hematocrit values of AZT-treated mice were lower than untreated controls throughout the study (data not shown). Two months after initiation of therapy and one month post inoculation with LP-BM5 MuLV, the

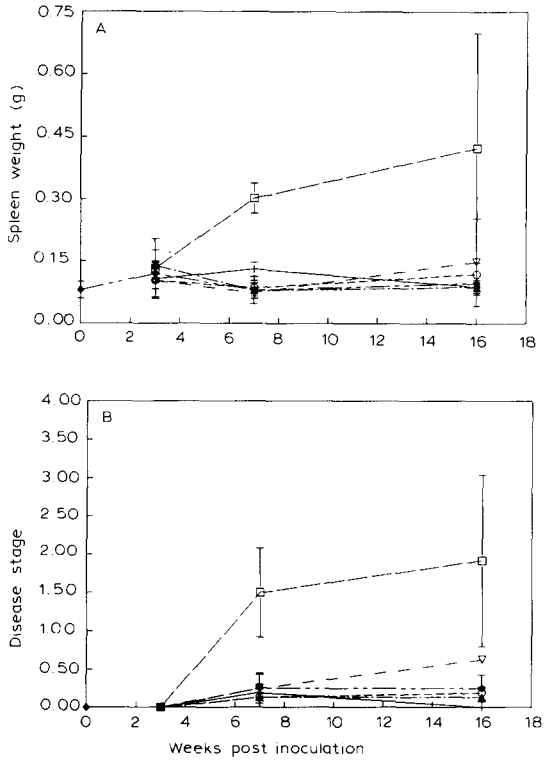

Fig. 3. Spleen weights and disease stage scores. Mice were killed at 3, 7, and 16 weeks post inoculation. (A) Spleen weights were measured immediately upon removal. (B) Tissues from mice euthanized at monthly intervals were fixed and scored as described. Results are the mean $\pm$ standard deviation of 4 animals per group; (◆—◆), control, uninfected mice; (□—□), LP-BM5 MuLV challenged mice; (○—○), 1 mg/ml AZT treated; (+—+), 1 mg/ml AZT treated, LP-BM5 MuLV; (▲—▲), 0.5 mg/ml AZT treated, LP-BM5 MuLV; (▽—▽), 0.1 mg/ml AZT treated, LP-BM5 MuLV.

TABLE 1

Plasma levels of AZT in mice after administration of AZT drinking water

AZT concentration in water	LP-BM5 MuLV	Weeks of administration		
		3	7	16
1.0 mg/ml	+	7.1	N	N
		1.2	2.7	N
		N	0.3	0.2
		N	0.3	1.5
		(2.1 ± 3.4)	(0.8 ± 1.3)	(0.4 ± 0.7)
1.0 mg/ml	−	N	0.7	15.8
		1.2	0.9	13.9
		N	11.6	0.4
		N	2.4	0.4
		(0.3 ± 0.6)	(3.9 ± 5.2)	(7.6 ± 8.4)
0.5 mg/ml	+	2.3	1.1	4.6
		N	N	N
		N	0.1	0.7
		N	N	N
		(0.6 ± 1.2)	(0.3 ± 0.5)	(1.3 ± 2.2)
0.1 mg/ml	+	N	3.9	N
		N	0.8	0.3
		N	N	1.1
		N	N	0.6
		(0 ± 0)	(1.2 ± 1.9)	(0.5 ± 0.5)

Mice were treated with the indicated concentrations of AZT in the drinking water, beginning 3 days prior to virus challenge and continuing until euthanasia at 3, 7 and 16 weeks p.i. Plasma levels of AZT (nmol/ml plasma) were measured on samples taken in the AM as described. Each value is that of an individual mouse. No AZT was detected in untreated mice. Values in parentheses are means ± the standard deviations. N, not detectable, a value below the limit of detection of the assay, 0.1 μM or 27 ng/ml.

mean hematocrit value of 4 of 5 AZT-treated mice killed at this time point was 36.6 ± 4.1%; one mouse had a hematocrit of 3.0% and aplastic bone marrow. The mean hematocrit for control animals killed concurrently was 49.0 ± 1.5%.

Having established that 1 mg/ml AZT in the drinking water beginning a month before virus challenge was effective in inhibiting disease and spread of ecotropic virus, a series of experiments were undertaken to examine if concentrations of AZT lower than 1.0 mg/ml might also be protective. Treatment with AZT, at concentrations of 1 mg/ml, 0.5 mg/ml and 0.1 mg/ml in the drinking water, was initiated three days prior to challenge with LP-BM5 MuLV and continued throughout the study. Plasma levels of AZT were measured in mice killed at 3, 7 or 16 weeks post LP-BM5 MuLV inoculation to determine if plasma AZT levels correlated with protective effect. Doses of 0.5 and 1 mg/ml AZT in the drinking water were effective in controlling disease, since both spleen weight and histopathology indicated no significant disease (Fig. 3). The lowest concentration examined, 0.1 mg/ml in drinking water, was

not as effective but still delayed disease progression. Consistent with the fact that mice drank AZT containing water at random times prior to sacrifice, AZT levels in the serum at the time of sacrifice showed a wide variation within groups. An example of the disparity in plasma levels is shown in Table 1, where while the highest concentrations were found in the 1 mg/ml group, levels were not directly correlated with the amount of drug in the water. No differences in plasma levels of AZT were detected between virus infected or control mice receiving 1 mg/ml AZT. Water consumption was similar for the groups. Control mice receiving 1 mg/ml AZT drank 5.11 ± 0.98 ml/mouse/day; infected mice receiving 1 mg/ml AZT drank 5.05 ± 1.03 ml/mouse/day. Untreated control mice consumed 5.35 ± 0.93 ml/mouse/day and untreated LP-BM5 MuLV infected mice drank 5.72 ± 1.34 ml/mouse/day. Infected mice treated at AZT concentrations of 0.1 mg/ml and 0.5 mg/ml AZT consumed respectively 5.49 ± 1.05 and 5.50 ± 1.09 ml/mouse/day. However, virus titers

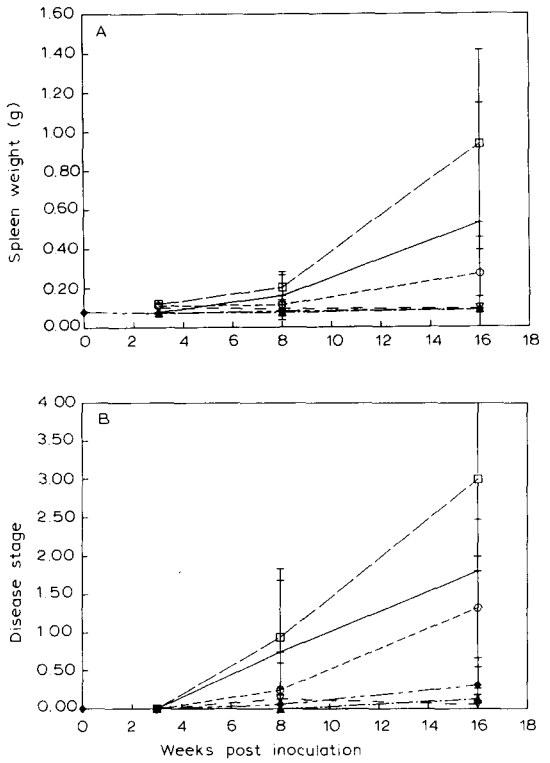

Fig. 4. Spleen weights and disease stage scores. Mice were killed at 3, 8, and 16 weeks post inoculation. (A) Spleen weights were measured immediately upon removal. (B) Tissues from mice euthanized at monthly intervals were fixed and scored as described. Results are the mean ± standard deviation of 4 animals per group; (◆—◆), control, uninfected mice (saline loaded Alzet pumps implanted); (□—□), LP-BM5 MuLV challenged mice; (▲—▲), 7 day AZT treated, uninfected mice; (▽—▽), 21 day AZT treated; (+—+), 7 day AZT treated, LP-BM5 MuLV; (○—○), 21 day AZT treated, LP-BM5 MuLV.

TABLE 2

Ecotropic virus expression in splenocytes from LP-BM5 MuLV challenged mice receiving AZT in the drinking water

AZT concentration in water	Weeks of administration		
	3	7	16
1 mg/ml	0	0	0
	0	6	0
	0	13	0
	0	0	0
	(0±0)	(5±6)	(0±0)
0.5 mg/ml	0	25	0
	0	0	0
	0	0	0
	0	0	0
	(0±0)	(6±12)	(0±0)
0.1 mg/ml	10	50	10
	0	100	2512
	0	0	1
	0	100	4
	(3±5)	(63±48)	(631±1253)
0	126	3162	3162
	316	6310	794
	13	1259	100
	398	5012	–
	(213±176)*	(3936±2203)*	(1352±1605)

Mice were treated with AZT at the indicated concentrations in drinking water, beginning 3 days prior to virus challenge and continuing until sacrifice at 3, 7, or 16 weeks p.i. Spleens were aseptically removed and the number of ecotropic virus infected cells (i.c.) was determined as described. No virus infected cells were detected in spleens from uninfected ($n = 12$) or uninfected AZT-treated ($n = 12$) control mice. Each value (no. of i.c. per 10^6 splenocytes) represents an individual mouse.

*Value different from other groups using Newman-Keuls Pairwise Comparison Test following ANOVA, $P \leqslant 0.05$.

inversely reflected the dose of AZT (Table 2). Virus was detected in the spleens of only 3 of 24 mice receiving 0.5 or 1 mg/ml AZT. In these three infected mice, the number of infected cells was about 100-fold lower than the number of infected splenocytes in untreated LP-BM5 infected mice. Eight of the twelve mice receiving 0.1 mg/ml in the drinking water had detectable virus. However, the number of infected splenocytes in these mice was at least 10-fold lower than the corresponding untreated infected mice.

Since administration of AZT in the drinking water was intermittent and dependent upon when the animal drank, continuous infusion via miniosmotic pumps was evaluated. Pharmacokinetic studies showed that ALZET 2001 pumps released a constant level of the drug for a period of 7 days and achieved steady state serum concentrations of approximately 1.50 μM, dependent primarily on the weight of the recipient (data not shown). One μM AZT had been shown to be greater than 10-fold higher than the dose necessary to prevent

LP-BM5 MuLV infection of susceptible cells in vitro. Pumps were implanted in mice 24 h prior to inoculation with LP-BM5 MuLV and removed on the seventh day. New pumps were inserted into some groups of mice on the seventh and 14th days. At three weeks post inoculation, virus infected cells were not detected in spleens of mice still receiving continuous infusion AZT, while infected cells were present in the spleens of mice which had AZT for only 7 days or untreated mice at that time (Table 3). Eight weeks post infection, 7 of 8 AZT treated mice were virus positive. By 16 weeks post challenge, all treated mice were virus positive. Nonetheless, those mice that had received AZT by continuous infusion for 3 weeks had significantly lower virus titers than the corresponding untreated infected mice. AZT, by continuous infusion for 21 days, delayed disease progression; spleen weights from these mice were similar to those of AZT-treated uninfected mice at 8 weeks and 3-fold lower than the

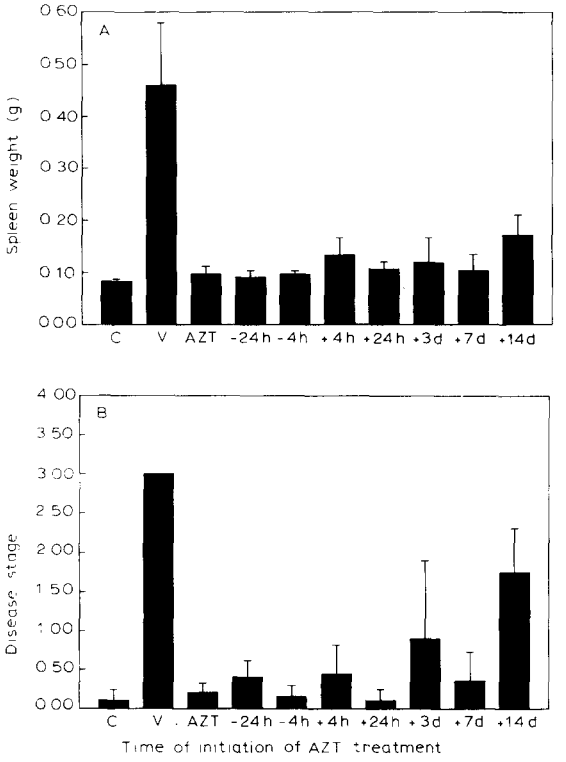

Fig. 5. Spleen weights and disease stage scores. Mice were killed at 2 months post inoculation. (A) Spleen weights were measured immediately upon removal. (B) Tissues from these mice were fixed and histopathologically scored as described. Results are the mean ± standard deviation of 4 animals per group; C, control, uninfected mice; V, LP-BM5 MuLV challenged mice; AZT, 1 mg/ml AZT treated; −24 h, AZT, 1 mg/ml 24 h prior to LP-BM5 MuLV; −4 h, AZT, 1 mg/ml 4 h prior to LP-BM5 MuLV; +4 h, AZT, 1 mg/ml 4 h after LP-BM5 MuLV; +24 h, AZT, 1 mg/ml 24 h after LP-BM5 MuLV; +3 d, AZT, 1 mg/ml 3 days after LP-BM5 MuLV; +7 d, AZT, 1 mg/ml 7 days after LP-BM5 MuLV; and +14 d, AZT, 1 mg/ml, 14 days after LP-BM5 MuLV.

untreated infected mice at 16 weeks. The stages of disease observed in the mice which had received AZT for 21 days were lower than the untreated infected mice at both 8 and 16 weeks (Fig. 4).

Since AZT was effective when therapy was initiated prior to virus challenge, a final study was undertaken to examine the efficacy of AZT when therapy was initiated after virus challenge. AZT, 1 mg per ml was administered as a single dose by oral gavage (0.25 ml) at 24 h and 4 h prior to and 4 h and 24 h post infection. Thereafter the mice received AZT in the drinking water at 1 mg/ml. Drinking water therapy was also started 3, 7 and 14 days after virus challenge in mice that had not received oral gavage. Assays of ecotropic virus expression in splenocytes from mice two months after LP-BM5 MuLV inoculation indicated that AZT treatment initiated anytime within a two week period was

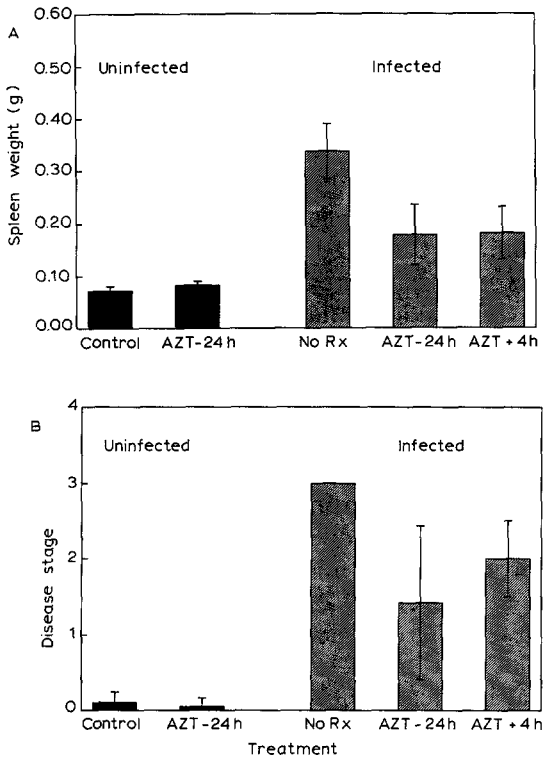

Fig. 6. Spleen weights and disease stage scores. Mice were treated with AZT, 1 mg/ml in drinking water beginning 24 h before or 4 h after virus challenge. Treatment continued for 21 days after inoculation and mice were killed at 2 months post inoculation. (A) Spleen weights were measured immediately upon removal. (B) Disease stage as determined histologically. Results are the mean ± standard deviation of at least 4 animals per group; Solid bars represent, control, uninfected mice labeled control and AZT, −24 h, uninfected mice treated with AZT, 1 mg/ml. Cross-hatched bars represent LP-BM5 MuLV-challenged mice; No Rx, indicates untreated LP-BM5 MuLV challenged mice; AZT, −24 h, indicates mice treated with AZT, 1 mg/ml, 24 h prior to challenge with LP-BM5 MuLV; AZT, +4 h, indicates mice treated with AZT, 1 mg/ml, 4 h after LP-BM5 MuLV challenge.

able to reduce virus load. No virus was present in the spleens of 4 of 5 mice receiving AZT therapy 24 h or 4 h prior to virus inoculation. Spleens of all four mice that had received AZT 4 h after virus inoculation were also virus free. In other groups where AZT treatment was initiated after virus challenge, virus-infected splenocytes were detected in all animals but the frequency of infected cells was significantly lower than in the untreated, infected mice. Spleen weights and disease stage scores suggest that AZT can prevent development of disease if therapy is initiated prior to LP-BM5 challenge and continued (Fig. 5). If this therapy is continued for only 21 days after virus inoculation, disease progresses, although slightly delayed, and the protective effects of AZT therapy are abrogated (Fig. 6). All spleens from these LP-BM5 MuLV inoculated AZT-treated mice were infected with ecotropic virus (data not shown).

Discussion

AZT was used as a test compound since it inhibits the process of reverse transcription, a molecular target common to HIV and MuLV, and one where the mechanism of inhibition has been shown to be similar in in vitro studies with purified enzymes (Furman et al., 1986; Furman and Barry, 1988; Balzarini et al., 1988). Since AZT inhibits replication prior to the integration of the MuLV provirus, we initially evaluated the ability of AZT to be used as a prophylactic agent. This study compared the efficacy of AZT using two routes of administration, oral via drinking water and subcutaneous constant infusion. Oral AZT was administered at 1.0, 0.5 and 0.1 mg/ml which results in a average daily dose of approximately 5, 2.5 and 0.5 mg AZT/mouse/day respectively while the continuous infusion dose was 0.6 mg AZT/mouse/day. HPLC measurements demonstrated that a miniosmotic pump containing AZT at 25 mg/ml maintained plasma levels of 1.5 μM AZT, while mice on drinking water regimens often had plasma concentrations below detection (<0.1 μM). Since both plasma and tissue pharmacokinetic studies indicate that the $t_{1/2}$ for AZT in C57BL/6 mice is approximately 20 min (Eiseman et al., in preparation) there is a marked difference in the level of AZT (and presumably AZT triphosphate) maintained at the sites of virus infection depending upon the route of administration of AZT.

The data indicate that both continuous infusion of AZT and drinking water regimens are effective in delaying disease progression. Continuous administration of AZT in the drinking water initiated one month prior to infection with LP-BM5 MuLV protected 13 of 15 (87%) mice inoculated with a lethal dose of LP-BM5 MuLV. Both of the non-protected, infected, AZT-treated mice had reduced numbers of infected splenocytes, indicative of a reduction in the virus load. Similarly, Ohnota et al. (1990), reported that oral AZT treatment (1 mg/ml in drinking water) prevented LP-BM5-induced immunodeficiency in C57BL/10 mice if treatment was initiated at the time of infection. Generally, the number of animals free of disease was proportional to AZT concentration

administered via the drinking water. Our studies using drinking water concentrations below 0.5 mg AZT/ml are in general agreement with post-exposure studies published by Basham et al. (1990). Basham and her co-workers reported increased survival and decreased virus load in C57BL/6 mice treated with 0.1 and 0.25 mg AZT/ml. AZT is effective in preventing or delaying the development of MAIDS if given continuously beginning prior to infection as a prophylactic agent. However, AZT was neither fully protective nor curative and it appears that antiviral therapy needs to be continued for an extended period of time, perhaps throughout the life of the animal. Continuous treatment with AZT beginning within 4 h after virus inoculation was able to protect mice from infection with LP-BM5 MuLV. If therapy was given for only 21 days following virus inoculation, by either constant infusion or ad libitum in the drinking water, the treatment was capable of delaying disease, but did not protect the animals from virus infection.

No ecotropic virus-infected cells were detected in spleens of 4 of 5 mice killed after three weeks of treatment with 0.1 mg/ml in drinking water. Similarly, maintenance of plasma AZT concentration of approximately 1.5 μM by constant infusion pumps also resulted in protection of splenocytes from infection at 3 weeks. The fact that virus was subsequently isolated from the spleens of similarly treated mice several weeks after cessation of AZT therapy suggests establishment of cellular reservoirs of infection during our attempts at prophylaxis with AZT. Since the macrophage is one of the first cell types infected with LP-BM5 MuLV (Mosier et al., 1988b), it may be that this cell type acts as a reservoir for the virus and once AZT therapy is stopped, this cell is able to transmit the infection either by cell to cell contact or by release of free virus particles.

Whatever the mechanism for disease progression after AZT prophylaxis, it is clear that the establishment of cellular reservoirs is not due to the drop in the serum AZT level below some minimal inhibitory concentration. AZT was protective at drinking water doses of 0.5 or 1 mg/ml, where AZT was not detectable in the plasma of 12 of 24 mice killed at 3, 7 or 16 weeks of therapy (Table 1). Further, infection occurred in mice whose plasma concentrations of AZT were maintained at approximately 1.5 μM by constant infusion (Table 3).

The administration of AZT for a limited time beginning prior to or shortly after exposure of mice to MuLV has been shown to prevent the establishment of infection and disease in several murine model systems. AZT alone or in combination with Interferon-α A/D has been shown to prevent Rauscher MuLV infection of adult BALB/c mice and Cas-Br-M MuLV infection of NFS/N mice (Ruprecht et al., 1987, 1988a, 1989; Bilello et al., 1990). Morrey et al. (1990) reported that oral or intraperitoneal administration of AZT for a maximum of 29 days post Friend virus infection of (B.10A $\times$ A/WySn)F_1 mice, inhibited splenomegaly, eliminated both plasma viremia and splenic infectious centers and resulted in 100% survival. Bilello et al. (1990) showed that AZT, at concentrations of 0.5 mg/ml and above in the drinking water, administered to lactating dams for 21 days, was sufficient to protect the pups

TABLE 3

Ecotropic virus expression in splenocytes of LP-BM5 MuLV challenged mice receiving AZT by constant infusion

AZT Rx	Weeks post inoculation		
No. of days	3	8	16
7	1	16	1995
	100	0	3
	50	1259	7943
	–	1589	316
	(50 ± 50)	(716 ± 829)	(2564 ± 3690)
21	0	159	1259
	0	794	1259
	0	126	1000
	0	63	50
	(0 ± 0)	(286 ± 341)	(892 ± 574)
–	631	158	3162
	1259	100	7943
	5	1584	3981
	500	2512	–
	(599 ± 516)	(1089 ± 1171)	(5029 ± 2557)

Mice were administered AZT (25 μg/h) by constant infusion from subcutaneously implanted ALZET miniosmotic pumps beginning 24 h prior to inoculation with LP-BM5 MuLV. Treatment was for the number of days indicated. Mice were killed 3, 8, and 16 weeks p.i. and spleens were removed aseptically. The number of splenocytes infected with ecotropic virus (no. of i.c./10^6 splenocytes) was determined as described in the methods. Individual animal values are presented and the group mean and standard deviation are indicated in parentheses. No ecotropic virus infected cells were detected in spleens of uninfected, treated ($n = 24$) and untreated ($n = 12$) mice. As controls saline or AZT loaded miniosmotic pumps were placed in both control and infected animals. In infected mice with pumps releasing saline there was no significant difference in any measurable parameter of disease progression. In uninfected control animals receiving AZT from an ALZET pump there was no difference in health status including hematocrit, which was normal.

from challenge with Cas-Br-M MuLV. While the above models suggest that AZT therapy may be of limited duration, the studies with LP-BM5 MuLV infection presented here do not support this conclusion. One possibility is that the differences observed between different murine models is dependent on the initial virus load and the cell types infected by the individual strains of MuLV. An alternative explanation for the differences in therapeutic efficacy of AZT between the murine models may be the immune competency of the host. While Rauscher MuLV infection is immunosuppressive, LP-BM5 induces AIDS-like functional defects in cellular and humoral immune responses. The differences in AZT protective effects may depend upon the ability of the host immune system to recognize the remaining virus or virus expressing cells and eliminate them. In the Rauscher model, it appears that there is an immune component to protection, since mice treated with AZT were protected from re-infection even after cessation of therapy (Ruprecht et al., 1988b). No antibody to LP-BM5 MuLV was detectable in C57BL/6 mice with MAIDS (Yetter, unpublished observation).

Our findings in the MAIDS model correlate well with the observed clinical experience with AZT in individuals infected with HIV (Fischl et al., 1987; Richman et al., 1987; Pizzo et al., 1988; Yarchoan et al., 1986). One limitation of AZT treatment in both mice and humans is hematopoietic toxicity (Richman et al., 1987; Bogliolo et al., 1988). In LP-BM5 infected mice, hematocrits were lower in the AZT treated mice throughout the studies employing 1 mg/ml in the drinking water, but the regimen was generally well tolerated. Treatment of mice with higher doses of AZT, i.e. 11.25 mg/mouse/day, resulted in aplasia, anemia and death and the depression in hematocrit was proportional to dose of AZT administered. No significant differences in hematocrits were observed in mice receiving continuous infusion AZT. Similar to observations with patients on AZT (Greenberg and Berger, 1990; Fisher and McPoland, 1989), hyperpigmentation was observed. All AZT-treated mice had increased pigmentation, primarily of the tail, ears and foot pads by 3 weeks of therapy. The therapeutic benefit of AZT in the treatment of both MAIDS and AIDS is delay in disease progression, presumably by control of virus replication and spread.

It should be emphasized that this study used a tightly controlled animal model of retroviral infection, MAIDS. Adapting MAIDS for use in in vivo antiviral screening required the use of a standard dose of virus sufficient to produce disease in 100% of the animals: mice of the same sex and strain with no exposure to exogenous pathogens and therefore little variability in immune competency. Until we are able to specifically quantitate the LP-BM5 defective disease-inducing virus and produce virus pools with a defined helper and defective virus population, the LP-BM5 system will not be readily standardized between laboratories. It is even more difficult to extrapolate results from studies with MAIDS to therapies for patients exposed to or infected with HIV. Nonetheless it appears that this model can provide an insight and guidance into the usefulness of single agent and perhaps more importantly combination antiviral therapy. The human data available does not allow determination of the amount of free virus or virus infected cells required to induce disease. Recent studies characterizing virus load in symptomatic and asymptomatic patients indicate that even within populations of patients with similar stages of illness the virus load in the plasma varies widely, however, increased levels of virus were generally associated with advanced disease (Ho et al., 1989; Coombs et al., 1989).

In conclusion, the implications of this study are: (i) AZT administered orally or by continuous infusion is an effective chemopreventive agent, (ii) initiation of AZT therapy prior to challenge with LP-BM5 MuLV is most effective, (iii) continuous treatment with AZT controls virus replication in a dose-dependent manner, and (iv) control of infection can prevent disease.

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