ABSTRACT
Due to similarities between human immunodeficiency virus and feline leukemia virus, the etiological agents of acquired immunodeficiency syndromes in humans and cats, the feline system was used as a model to conduct preliminary investigations as to the efficacy of the thymidine analogue 3'-azido-3'-deoxythymidine (AZT) as a therapeutic and preventive agent against retroviruses. In vitro evaluations of AZT cytotoxicity and its antiviral effects were conducted. Subsequently, 50 6-week-old specific pathogen free kittens were inoculated with a highly immunosuppressive strain of Rickard-Feline Leukemia Virus. These cats were randomly subdivided into smaller groups with initiation of AZT treatment at variable times postinfection. All animals were periodically monitored for circulating infectious virus particles and virus-neutralizing antibodies. Their clinical condition was closely followed throughout the 6-week AZT treatment phase and for several months thereafter.

The results indicate that AZT prevents retrovirus infection if administered immediately following virus exposure, and may also reduce retrovirus replication if administered to previously infected animals. Some of the treated cats developed neutralizing antibodies against the virus and became resistant to subsequent viral challenge. Future trials with this drug, both for the prevention and treatment of retroviral diseases in humans and animals, are warranted.

INTRODUCTION
Acquired immune deficiency syndrome (AIDS) first reported in 1981 (1, 2), has been etiologically linked to the human immunodeficiency virus (HIV or HTLV-III/LAV), and well characterized clinically, immunologically, and epidemiologically (3–5 for review). AIDS patients develop lymphopenia with marked reduction of T4 helper cells and a consequent suppression of immune system function which greatly increases their susceptibility to opportunistic infections and unusual malignancies (5). The control of this disease is especially difficult due to the extended period of time between infection and the first appearance of clinically recognizable symptoms during which the virus can be transmitted.

Many drugs are currently under investigation as possible agents for the treatment of AIDS, e.g., HPA-23 (6), ribavirin (7), interferon (8), and phosphonoformate (9). One such agent, AZT\(^3\) has been shown to be a potent in vitro inhibitor of HTLV-III replication and cytopathic effect in human cells (10). AZT has also been shown to be an effective inhibitor of murine and feline retrovirus replication in vitro (11, 12).

AZT, a thymidine analogue, was synthesized as described elsewhere (13). Briefly, the 3'-hydroxy group of thymidine was substituted by an azido group (N\(^3\)). Within the infected cell the compound is converted to a triphosphate form which is used by retroviral reverse transcriptase and incorporated into the DNA transcript. There, due to the 3'-substitution, AZT blocks further DNA chain elongation preventing viral DNA insertion into the host-cell's genome and consequent viral replication (14, 15).

AZT is now being used in clinical trials in AIDS patients. The results reported so far indicate that treated patients experience a significant improvement of their clinical condition and a partial recovery of their immune functions (14). A decrease in the mortality rate of treated patients as compared to placebo controls has also been observed (3, 14).

FeLV, a naturally occurring retrovirus discovered in 1969 by W. F. H. Jarrett and associates (16), is one of the most thoroughly studied retroviruses affecting outbred species. It is horizontally transmitted and widespread among domestic cats. Over 1,000,000 cats in the United States, representing approximately 2% of the total cat population, are infected with FeLV, the causative agent of the infectious disease responsible for most deaths within this species (17, 18). FeLV causes both lymphoproliferative diseases, primarily T-cell lymphomas and marked immunosuppression. Recent studies show that most FeLV-infected cats die as a direct consequence of immune system impairment. This FeLV-associated immunodeficiency is very similar to human AIDS (17–19). FeLV-infected animals remain highly viremic and virus particles can be accurately detected and titrated with focus induction assays, making the feline model very attractive for retroviral studies.

A particularly immunosuppressive isolate of FeLV biologically cloned from the Rickard strain has been used in our experiments. Here we report the results obtained from experiments in which FeLV-infected cats were treated with AZT at different stages of experimental infection.

MATERIALS AND METHODS
FeLV Strain
The virus used was biologically cloned in animals from Rickard FeLV strain. Briefly, several 10-fold viral dilutions were made and each dilution was inoculated into five SPF kittens. Virus isolation was performed from the animals receiving the highest viral dilution (10\(^{-5}\)) that caused persistent viremia, but no detectable tumor development 6 months postinoculation. The virus isolate was maintained in normal cat embryo cells (NCE-232).

Cell Lines
81C/sarcoma-positive/leukemia-negative cells were obtained from Dr. P. Fischinger (20) and cultivated in McCoy's 5a medium (GIBCO, 320-6600) supplemented with 15% fetal bovine serum and 1.5% antibiotic/antimycotic (GIBCO, 600-5240).

Experimental Animals
All kittens used were obtained from the Cornell SPF cat colony. The 50 weanling kittens were randomly divided and housed two per Horsfall cage.
AZT

3'-Azido-3'-deoxythymidine (M, 267.64) was provided by Burroughs-Wellcome Co. The AZT used in the in vivo assays was dissolved in phosphate-buffered saline, pH 7.2, to a final concentration of 20 mg/ml and sterilized by filtration through 0.22-µm Nalgene filters. AZT used in the in vitro assays was diluted in McCoy's 5A medium to the following concentrations: 100, 50, 10, 5, 1, 0.5, 0.1, and 0.05 µM, and sterilized by filtration.

In Vitro Assays

AZT Toxicity. To assess possible cytotoxic effects of AZT in vitro 81C cells were cultivated for 12 days in McCoy's medium containing the previously described drug concentrations. Cells (3 x 10⁴)/well were seeded in a 24-well tissue culture plate (Costar 3524). Two wells were used per drug concentration tested, while four wells, containing media but no AZT, served as controls.

Assay for Viral Replication Inhibitory Effects. 81C cells (2 x 10⁴) were seeded into 60-mm diameter, 2-mm gridded plates and incubated at 37°C (7% CO₂) for 8 h in polybrene (1 µg/ml) 15% fetal bovine serum McCoy's 5A medium. Medium was removed from the plates and infection with an FeLV dilution, previously titrated and known to induce approximately 100 foci/plate, was allowed to take place for 45 min at 37°C. Medium containing AZT at the concentrations described was then added to the plates (two plates per drug concentration). Four plates served as virus controls (infected, but with no AZT added to the media). Four other plates served as uninfected controls. All plates were incubated for 12 days at 37°C, 7% CO₂. Medium was changed every 3 days while constant AZT concentrations were maintained. All plates were scored on the 12th day postinfection by counting the number of foci.

AZT Direct Antiviral Effect. To assess the possibility that AZT directly inactivates cell-free virus particles, 100 FIU of FeLV in 0.1 ml of Liebovitz (L-15) medium was incubated for 2 h at room temperature with 0.1 ml McCoy's medium containing the different AZT concentrations, prior to infection of 81C plates (as described before). Two plates were used per drug concentration. After viral infection occurred cells were washed twice with plain McCoy's medium and maintained in drug-free media renewed every 3 days. Following a 12-day incubation period the plates were scored for number of foci.

In Vivo Assays

Once the pharmacokinetics of AZT in the cat system were evaluated, the in vivo assays were initiated according to the following protocol: treatment group A consisted of 12 animals divided into three groups each receiving AZT s.c. at a dose of 20 mg/kg body weight, twice a day beginning 1 h (group A1), 7 days (group A2), and 28 days (group A3) postinfection. Treatment group B consisted of 12 kittens each receiving 10 mg/kg body weight twice a day in the same therapy regimen as group A. These subgroups were designated B1, B2, and B3, respectively. Group C consisted of 10 kittens that began treatment 3 days after infection receiving 20 mg/kg body weight 3 times a day. Twelve kittens were used as virus controls (group Vc, untreated). Two uninfected animals served as drug controls at each dosage level. Pretreatment blood samples were collected and body weights were recorded. AZT dosages were adjusted weekly according to body weight. Blood samples were drawn every 14 days to evaluate viremia and serum antibodies. All animals were treated for a period of 42 days and monitored for viremia and neutralizing antibodies for 120 days.

Virus Infection

All kittens, with the exception of the drug controls, were infected prior to the initiation of AZT treatment, by a s.c. inoculation of 2 x 10⁸ FIU of FeLV in 1 ml sterile L-15 medium. Virus challenge with 4 x 10⁸ FIU (double the initial dose) was performed on day 120 postinfection in all cats that remained virus-negative and had developed NA.

ASSAY FOR AID CHEMOTHERAPY

Viremia levels and other virus titrations were obtained with 81C cells as described elsewhere (20, 21).

Assay for Evaluation of Serum Neutralizing Antibodies

Serum neutralizations were performed biweekly using serum samples from all tested animals. Samples (0.2 ml) of different test serum dilutions (undiluted, 1:10, 1:20, and 1:40) in L-15 medium were incubated with 100 FIU of FeLV for a period of 45 min at 37°C prior to inoculation onto 81C cells. Two plates were made per serum dilution. Four plates using undiluted normal SPF cat serum served as negative controls. Four virus control plates were also made as described before. After 12 days all plates were read and the number of foci scored. Serum dilutions causing 50% reduction of the foci numbers counted in SPF sera control plates were considered positive.

RESULTS

In Vitro

Assay for AZT Toxicity. The in vitro experiments showed that even at the highest AZT concentrations tested, the 81C cells grew normally as compared to untreated controls and there was no indication of toxicity.

Protective Effect of AZT on Infected Cat Cells. (See Fig. 1). The ability of AZT to reduce or abrogate virus replication in 81C cells was assessed in this experiment. Total inhibition was obtained at drug concentrations of 100 and 50 µM while partial inhibition was observed at concentrations of 10, 5, 1, and 0.5 µM. No effect was detected at lower concentrations (0.1 and 0.05 µM). Data plotted in Fig. 1 were obtained by averaging the number of foci counted on the two plates made per drug concentration.

AZT Direct Antiviral Effect. The possible direct effect of AZT to inactivate free virus particles was evaluated by incubating a known titer of FIU of FeLV with several concentrations of AZT for 2 h prior to infection of 81C cells. The results demonstrated that none of the AZT concentrations tested had any direct effect on cell-free virions. No significant difference was seen between the number of foci scored in treated virus-infected plates and nontreated controls. (data not shown).

In Vivo

AZT appears to be nontoxic in vivo at the doses used (10 and 20 mg/kg) as assessed by weight gain and clinical examination...
of four AZT-treated uninfected kittens. However, three kittens in group C from the same litter treated with 20 mg/kg (three times a day) showed anorexia, vomiting, and icteric mucosae after 40 days of treatment. These could have been signs of idiosyncratic hepatotoxicity (see “Discussion”).

In the first groups, A1 and B1, where treatment began 1 h PI with high and low dosages of AZT, all kittens remained FeLV-negative throughout the experiment regardless of the drug dosage used (Fig. 2). No clinical disease was detected in any of the A1 and B1 cats. One animal out of eight developed NA and was resistant to the FeLV challenge performed 120 days PI.

For the kittens treated 7 days PI results appear to be dose related (see Table 1). Of the four animals receiving an AZT dosage of 20 mg/kg (group A2), two maintained their virus-negative status throughout the experiment, one became persistently viremic 11 weeks PI (5 weeks after treatment had been discontinued), and one passed through a period of transient viremia detected on day 28 PI and subsequently became virus-negative. The two cats that remained negative and the transient viremic cat developed neutralizing antibodies and resisted subsequent virus challenge showing no signs of clinic disease. Of the kittens treated with the lower dose (group B2), two were transiently viremic at week 4 but returned to a negative status at week 6 and remained negative thereafter, showing high levels of neutralizing antibodies and resisting virus challenge. The other two animals in this group became persistently viremic 28 days after virus inoculation showing evidence of progressive disease. One of them died 15 weeks PI.

All cats in groups A3 and B3 were viremic by the time treatment was initiated, 28 days PI, and remained viremic throughout the experiment period. Levels of circulating virus particles in these cats’ plasma remained relatively low during AZT treatment (1–2 × 10⁸ FIU/ml of plasma in group A3 and 2–3 × 10⁸ FIU/ml of plasma in group B3), but increased (up to 1 × 10⁹ FIU/ml plasma) within 30 days following termination of treatment (Fig. 2).

Of the 10 kittens in group C (receiving 20 mg/kg AZT, three times a day, starting 3 days PI), five remained virus-negative, two were transiently viremic at week 4, and three became persistently viremic at weeks 6 and 8. These three kittens were all from the same litter. Six animals in this group developed neutralizing antibodies and resisted the virus challenge.

Eleven of the 12 virus control kittens, group Vc, became highly viremic. Six of them died between weeks 6 and 15 PI, showing marked debilitation. The remaining kitten in group Vc (No. 634) exhibited transient viremia by week 4 but became virus-negative by week 6 developing neutralizing antibodies. It was the only case of spontaneous reversal of viremia.

Neutralizing Antibodies

One cat out of eight that received AZT treatment 1 h after infection showed NA (titer 1:10) by week 15 PI. Of a total of 18 cats in which AZT treatment started 3 or 7 days PI (groups A2, B2, and C), 11 developed NA (titers >1:20) first detected at week 6 (Table 1). All cats that had NA resisted the virus challenge performed 120 days PI. Further monitoring of these animals has not evidenced viremia.

None of the cats in which treatment started 28 days after viral inoculation (A3 and B3) was found to have neutralizing antibodies.

DISCUSSION

AZT is the best drug candidate tested so far to be used for the therapy of AIDS and other retrovirus-related diseases in humans and animals (3, 14).

The results obtained in our in vitro assays showed that AZT used in concentrations above 50 μM in the tissue culture media is able to completely inhibit FeLV replication in 81C cells infected 45 min to 1 h prior to treatment. Lower concentrations (0.5–10 μM) caused partial inhibition. These results agreed with those reported by Hardy et al. from a similar experiment using a different cell line (12).

The in vivo results showed that the time after infection when AZT administration was initiated was crucial for the outcome of the therapy. When treatment began 1 h after virus incula-

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*L. Tavares, C. Roniker, K. Johnston, S. Nusinoff Lehrman, and F. de Noronha, unpublished observations.
tion, both AZT doses used were effective in abrogating viral replication, since the eight animals in groups A1 and B1 remained negative throughout the experiment. On the other hand, results from groups A3 and B3, in which therapy was initiated 28 days PI when all animals had already shown viremia, proved that AZT therapy did not prevent progression of infection. However, the levels of viremia were kept much lower than in untreated cats (Fig. 2) during the 6 weeks of therapy, but increased immediately after treatment was discontinued.

Interesting results were obtained in groups C, A2, and B2 in which 18 previously infected animals began treatment 3 days (10 kittens in group C) and 7 days PI (four kittens in group A2 and four in group B2). Dosage level appears to be important in these groups. Of the 10 animals in group C treated with the highest dose regimen (20 mg/kg, three times a day), seven were virus free by the end of the treatment period and six of these had persistently high titers of circulating neutralizing antibodies. All have remained virus-negative to the present time. Notably, the three kittens in this group that became viremic at weeks 6 and 8 PI (end of AZT therapy) were all from the same litter and showed symptoms of liver dysfunction. These facts suggest a genetically related susceptibility to either the drug or the virus.

Cats in groups A2 (three of four) and B2 (two of four) that were virus-negative by the end of the therapy regimen and remained negative after the virus challenge developed neutralizing antibodies with titers greater than 1:20 detected for the first time at week 6 PI (Table 1). These results are in accordance with previous studies in which NA titers above 1:10 protected the animals from subsequent FeLV exposure (18). The importance of neutralizing antibodies for FeLV resistance has been well established in studies performed previously at this laboratory. The sequence of events described is: infection of local lymphoid tissue associated with the inoculation site, infection of circulation mononuclear cells, propagation to systemic lymphoid tissues. Virus replication can be detected in bone marrow cells 14–21 days after infection. After this point, infection is amplified by spreading to circulating leukocytes and platelets and viremia becomes well established propagating infection to epithelial tissues (28–56 days after infection).

Correlating these facts with the results from the present experiment permits postulation that the crucial point for reversibility of FeLV-induced leukemia is infection of bone marrow stem cells. Once this phase is reached, viral genome integrates into stem cell DNA, and all lymphoid cells originating from these precursors will be FeLV infected. When AZT treatment starts before the bone marrow phase, AZT is able to prevent the normal evolution of the infection by blocking reverse transcription from viral RNA to DNA in infected cells.

The viral antigens released during early stages by infected local and circulating lymphocytes (1–14 days after infection) appear to stimulate the immune system to produce neutralizing antibodies. Since AZT protects bone marrow cells over a period of 6 weeks (treatment duration) the immune system has time to recognize and eliminate infected cells and infectious virus particles, thereby mounting an immune response that protects the animal from subsequent FeLV challenge. AZT treatment 1 h PI results in a prophylactic rather than therapeutic effect, blocking initial virus replication, thus impeding the antigenic stimulation required for a competent immune response.

Despite the limitations involved in extrapolating data from animal models to humans, the results of these preliminary studies provide a promising basis for further trials using AZT either as a prophylactic or therapeutic agent against AIDS and other retroviral diseases in humans and animals.

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