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Suppression of retroviral propagation and disease by suramin in murine systems

(reverse transcriptase inhibitors/antiretroviral therapy/mouse tissue culture cell lines/mice)

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ABSTRACT **Retroviral propagation crucially depends on reverse transcriptase (RT). We have developed murine models to test the biological effectiveness of the RT inhibitor suramin. The drug was active in our assay system, which includes (*i*) inhibition of RT activity in the murine T-cell tropic virus SL3-3 and Rauscher murine leukemia virus (MuLV), (*ii*) inhibition of plaque formation in the XC plaque assay, (*iii*) inhibition of viral infection of cultured murine T cells, and (*iv*) inhibition of splenomegaly induced by Rauscher MuLV in BALB/c mice. Suramin decreases viral titers significantly, even if started 36 hr after infection. Viral titers and number of infected cells increased to control levels after removal of the drug. BALB/c mice treated i.v. with 40 mg of suramin per kg twice per week following infection with Rauscher MuLV showed a 35% decrease in splenomegaly. Suramin is an active antiretroviral agent whose effect on retroviral propagation is reversible. We conclude that it acts as a virustatic drug and that long-term administration of suramin will be necessary if it is used for experimental treatment of human retroviral illnesses such as the acquired immune deficiency syndrome.**

The importance of retroviruses in inducing neoplasia and immunosuppression in animal species has long been recognized (1). Their role in human diseases is just beginning to be understood. The human T-cell leukemia virus type I (HTLV-I) has been causally linked to certain human T-cell leukemias and lymphomas (2). The virus was first isolated from a patient thought to have mycosis fungoides (3) and is endemic in certain areas of the Caribbean islands (4, 5) and Japan (6, 7). HTLV-I-positive leukemia/lymphoma has a grave prognosis, and conventional chemotherapeutic regimens are ineffective in giving long-term survival (8). The human T-lymphotropic virus type III (HTLV-III/LAV) (9, 10) is believed to cause the acquired immune deficiency syndrome (AIDS) (11). Like HTLV-I-positive T-cell malignancies, fully developed AIDS has a grave prognosis, and to date no curative therapy exists (12). The number of cases has been rising steadily.

HTLV-III can infect the brains of some AIDS patients (13). In tissue culture, it can grow not only in T-helper cells (9, 10, 14) but also in monocytic, endothelial, and glioblastoma cell lines (unpublished data). Such cells could represent a natural reservoir in virus-positive individuals.

Clearly, breaking the viral life cycle by therapeutic interventions could be a major step toward stemming retroviral epidemics. Pharmacological agents designed to interfere with retroviral functions such as reverse transcription could be important therapeutic means, possibly together with immunological reagents. Effective drugs need to have a high therapeutic index and little or no depression of bone marrow-derived cells, especially granulocytes. Presently, the major causes of death in AIDS patients are severe infections due to the lack of cellular immunity (15, 16). Most patients, however, do not present with bacterial sepsis like neutropenic cancer patients following cytotoxic chemotherapy.

The antitrypanosomal drug suramin (17) seems to be a candidate antiretroviral drug. De Clercq found that it reversibly inhibits the reverse transcriptase (RT) activity of avian and murine viruses (18). In a recent report, suramin inhibited the cytopathic effects of HTLV-III on T-helper cells cocultivated with a virus-producing cell line (19). Most importantly, there was little cytotoxicity at suramin levels able to prevent cytopathic effects. Suramin has, however, shown renal toxicity in humans treated for trypanosomiasis (17). This may prevent it from becoming a drug useful in the treatment of human retroviral diseases. Analogues of suramin with diminished renal toxicity may have to be tested. What is clearly needed is a screening procedure that allows rapid detection of useful antiretroviral agents, first by *in vitro* analysis. Agents that pass this first round would then be selected for analysis in animals.

We report the systematic evaluation of suramin in murine retroviral systems *in vitro* and *in vivo*, using the T-cell tropic virus SL3-3 (20) and the erythrotropic Rauscher murine leukemia virus (MuLV) (21). This latter agent was chosen for its short latent period for disease induction. Susceptible mice develop palpable splenomegaly within 2 weeks and usually succumb to the leukemia in 30–40 days. The degree of splenomegaly is proportional to the viral titer (22). The short latent period allows rapid determination of effective dosage. Biological effectiveness against T-cell tropic retroviruses can be studied in mice susceptible to SL3-3-induced leukemia/lymphoma. Onset of disease due to SL3-3 typically occurs between 60 and 90 days following injection of susceptible newborn mice (23, 24). The SL3-3 virus is of particular interest since chronic viremia precedes development of T-cell lymphoma. Agents capable of significant reduction of viremia and development of lymphoma due to SL3-3 should be active against other retroviruses with T-cell tropism and longer latent periods.

MATERIALS AND METHODS

Cell Lines. SC-1 and XC cells, obtained from M. Cloyd (Duke University), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. NIH 3T3 cells were grown in DMEM supplemented with 10% calf serum. Murine L691 cells, obtained from N. Rosenberg (Tufts University), were grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

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Abbreviations: RT, reverse transcriptase; HTLV, human T-cell leukemia virus; MuLV, murine leukemia virus; AIDS, acquired immune deficiency syndrome; pfu, plaque-forming units.

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Viruses. SL3-3 virus (20) was prepared by transfecting the cloned provirus (23) into NIH 3T3 cells. Tissue culture supernatant was stored at −70°C after filtration through a 0.45-μm filter. Rauscher MuLV, strain RVB3 (25), was provided by M. Strand (Johns Hopkins University). Virus stocks were prepared by injecting freshly thawed virus i.v. into 6-week-old BALB/c mice, which were sacrificed on day 20. Single cell suspensions of splenocytes were prepared in DMEM with 20% fetal calf serum. After spinning out the cells, the supernatant was passed through a 0.45-μm filter. Viral titers were assayed by the XC plaque assay.

XC Plaque Assay. The test was carried out as described (26), except that viral infections were carried out in the presence of 8 μg of Polybrene (Aldrich) per ml. Viral titers were calculated from an average of three dishes.

XC Infectious Center Assay (27). The test was carried out as was the XC plaque assay described above except that cells infected with murine ecotropic viruses were added to the SC-1 cells.

RT Assays. Crude virus preparations were analyzed for RT activity by using the procedure of Roy-Burman *et al.* (28). Suramin was obtained from the National Cancer Institute.

Granulocyte/Macrophage Colony-Forming Unit Assay of Mouse Bone Marrow Cells. Bone marrow cells were flushed from the femurs and tibias of 5-week-old BALB/c mice by using Iscove's modified Dulbecco's medium containing 20% fetal calf serum, antibiotics, 4 mM L-glutamine, and 100 units of heparin per ml. The cells were purified by a Ficoll/Hypaque gradient, washed three times in fresh medium, and plated by using the method of Cannistra *et al.* (29). Various concentrations of suramin were assayed in quadruplicate. Colonies were counted at 7 and 14 days.

***In Vivo* Testing in BALB/c Mice.** Five- to 7-week-old female BALB/c mice (The Jackson Laboratory) were injected in groups of 10; as controls, 19 mice received virus only, 10 mice were left untreated, and 10 mice received drug only. On day 0, the animals received either 0.25 ml of virus [titer, 6.7×10^4 plaque-forming units (pfu)/ml] or normal saline i.v. Four hours later, suramin or saline was given i.v. and was continued twice weekly. On day 20, all mice were sacrificed. Blood was collected by cardiac puncture, and pooled heparinized plasma was stored at −70°C until suramin levels were determined. Spleen weights were obtained for each animal.

Determination of Suramin Levels in Pooled Mouse Plasma. Suramin levels were analyzed in pooled heparinized mouse plasma by using the method of Klecker *et al.* (30).

RESULTS

Suramin was analyzed systematically for effectiveness in the following battery of tests: (*i*) inhibition of RT activity of the murine T-cell tropic virus SL3-3 and of Rauscher MuLV, (*ii*) inhibition of plaque formation in the XC plaque assay (26), (*iii*) inhibition of viral infection of murine T cells by SL3-3, and (*iv*) inhibition of splenomegaly induced by Rauscher MuLV in BALB/c mice.

Inhibition of RT Activity by Suramin. The effect of suramin on RT activity of SL3-3 virus and Rauscher MuLV is shown in Fig. 1. At a concentration of 6.3 μg of suramin per ml, 50% of the enzymatic activity was lost.

Prevention of Plaque Formation of SL3-3 Virus in the XC Plaque Assay in the Presence of Suramin. Murine SC-1 fibroblasts were pretreated with various concentrations of suramin, infected, and grown to confluency in the presence of the drug. Suramin was then washed out, and the SC-1 cells were killed with UV light. XC cells were added and plaques were scored 3 days later. In parallel dishes uninfected SC-1 cells were grown in various concentrations of suramin to determine cytotoxicity. The results are shown in Fig. 2. The

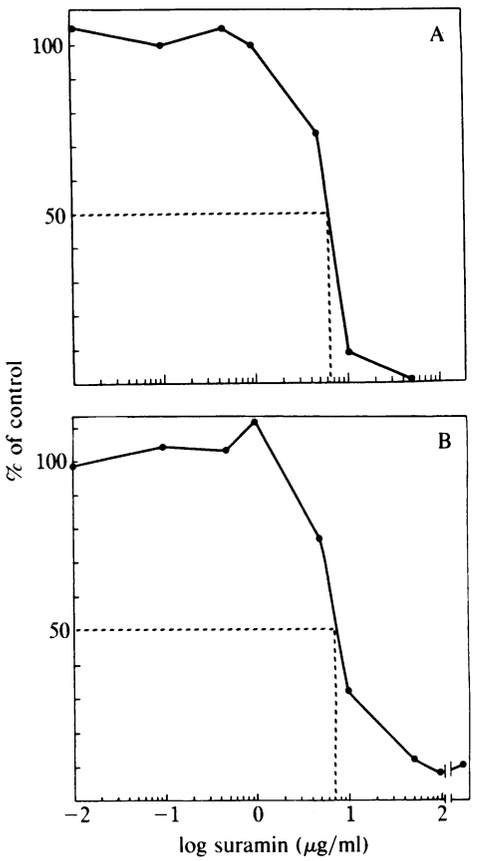

FIG. 1. Titration of suramin by using crude preparations of SL3-3 virus (*A*) or Rauscher MuLV (*B*) in the RT assay (28). Detergent-treated virus was incubated with various concentrations of suramin. The reaction mixture was incubated for 1 hr. The percentage of remaining activity is plotted against the concentration of suramin in μg/ml.

cells infected in the absence of suramin yielded a viral titer of 3.5×10^5 pfu/ml. At 50 μg/ml, the titer was reduced to 6×10^2 pfu/ml, and no plaques were detected at 100 μg of suramin per ml. Fig. 2*B* shows the effect of the various dose levels on cell number.

Inhibition of SL3-3 Viral Infection of Murine T Cells. Fig. 3 shows the effect on virus titers when L691 cells were infected in the presence of suramin. Cells were passed to a density of 5×10^4 cells per ml 24 hr before viral infection, and various concentrations of suramin were added. The cells were infected with SL3-3 virus in the presence of appropriate suramin concentrations and Polybrene at 4 μg/ml for 3 hr, washed three times, and grown in the continued presence of the drug for 10 days. Tissue culture supernatant was assayed for RT activity and titrated for infectious virus particles in the XC plaque assay. Supernatants from uninfected L691 cells and L691 cells infected with SL3-3 virus without suramin served as controls. Pretreatment and continuous presence of suramin led to a 2–3 logarithmic decrease in viral titer at 100 μg of suramin per ml. Inhibition of plaque formation in the XC plaque assay by suramin (Fig. 2) correlated with the suppression of viral titers when T cells were infected in the presence of suramin (Fig. 3). The somewhat greater inhibitory effect of suramin on suppressing titers of infected SC-1 cells may be explained by higher intracellular concentration of the drug. Suramin is thought to be taken up by cells in protein–suramin complexes. In animals it becomes concentrated in lysosomes of macrophages (17).

Next, we determined whether suramin must be present continuously after viral infection or whether high concentrations of suramin present only at the time of infection and

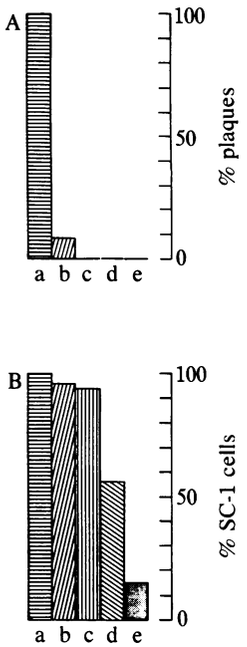

FIG. 2. (*A*) Inhibition of plaque formation in the XC plaque assay by suramin. SC-1 cells (1×10^5 per 60-mm dish) were pretreated for 24 hr with various concentrations of suramin (a, no suramin; b, 10 μg/ml; c, 50 μg/ml; d, 100 μg/ml; and e, 300 μg/ml) and infected with SL3-3 virus. Suramin was continued until UV killing of the SC-1 cells. The XC plaque assay was then continued. The number of plaques obtained at the various suramin concentrations is expressed as percentage of plaques obtained in the absence of suramin (a). (*B*) Analysis of cytotoxicity. SC-1 cells were treated as described above, trypsinized when control cells were confluent, and counted after trypan blue staining. The number of viable cells is expressed as percentage of untreated control cells (a).

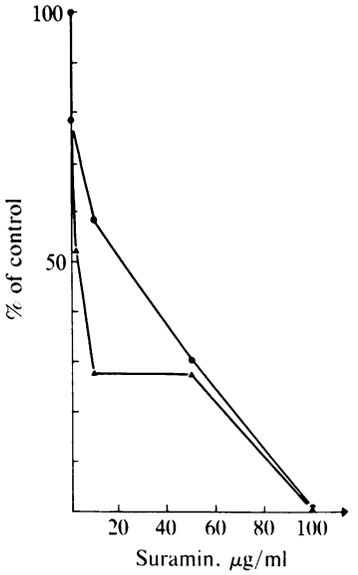

FIG. 3. Inhibition of SL3-3 viral infection of murine T cells. L691 cells were incubated for 24 hr at various concentrations of suramin, infected with SL3-3 virus in the presence of suramin and Polybrene at 4 μg/ml, washed three times, and grown for 10 days in suramin. Virus particles were then spun out and assayed for RT activity. The percentage of remaining enzyme activity is plotted against the concentration of suramin in μg/ml (▲). Various dilutions of tissue culture supernatant from each suramin concentration were tested for infectious virus in the XC plaque assay (26). Viral titers are expressed as percentage of pfu in supernatant from L691 cells infected in the absence of suramin (●).

immediately thereafter are sufficient to prevent viral infection of T cells. L691 cells were pretreated for 24 hr with 100 μg of suramin per ml followed by infection with SL3-3 virus in the presence of the same concentration of the drug. Uninfected L691 cells and L691 cells infected with the virus in the absence of suramin served as controls. When the supernatant of the control cells infected in the absence of suramin 17 days later showed a high level of RT activity, the number of virus-positive cells was assayed in the XC infectious center assay. Suramin-treated cells were washed to remove the drug before they were added to SC-1 cells. The results are shown in Fig. 4*A*. Sixty percent of the positive control L691 cells were virus positive, whereas infection of L691 cells in the presence of 100 μg of suramin per ml yielded 0.09% virus-positive cells. Suramin was removed from an aliquot of these latter cells, and all cultures were incubated again. Three days after removal of suramin, the supernatant of the L691 cells initially infected in the presence of 100 μg of suramin per ml showed very high RT activity. XC infectious center assays performed subsequently showed a dramatic increase in the number of virus-positive cells (Fig. 4*B*). To confirm these results, L691 cells were pretreated with various concentrations of suramin and infected in the presence of the drug as described above. Three days after viral infection, suramin was washed out. RT activities measured 10 days later were in the same range as those of control samples infected in the absence of suramin. Viral titers of tissue culture supernatants of L691 cells pretreated and infected in the presence of suramin at 100 μg/ml for 3 days followed by growth in regular medium for 1 week were virtually identical to those of control cultures infected in the absence of suramin (data not shown). We conclude that only very few cells become infected by high titers of virus in the presence of suramin, but that suramin must be present continuously to be effective. Thus, suramin is not a virucidal but rather a virustatic drug.

Effect of Suramin After Viral Infection of T cells. L691 cells

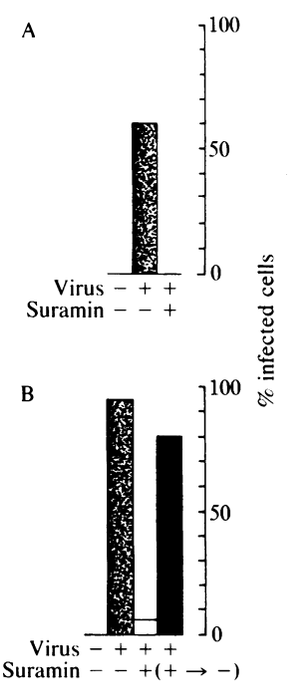

FIG. 4. (*A*) XC infectious center assay (27) of L691 cells infected with SL3-3 virus (stippled bar), uninfected L691 cells (left), and L691 cells infected in the presence of 100 μg of suramin per ml (right). The percentage of infected cells is shown. (*B*) Same as *A*. Suramin was removed from an aliquot of L691 cells infected in the presence of 100 μg of suramin per ml (black bar), and all cultures were assayed again for infected cells 6 days later.

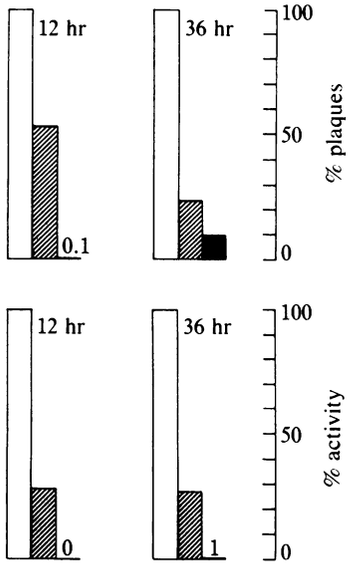

FIG. 5. Effect of suramin on viral titer and RT as a function of suramin concentration and time interval between infection and initiation of treatment. L691 cells were infected with SL3-3 virus in the presence of Polybrene at 4 μg/ml for 3 hr. Suramin was added at 10 μg/ml (striped bars) or 100 μg/ml (solid bars) 12 hr (left) or 36 hr (right) after viral infection. The cells were grown for 10 days in suramin. Open bars show control samples treated with virus only. Tissue culture supernatants were tested for plaque formation in the XC plaque assay (top panels) and for RT activity (bottom panels).

were infected in the presence of Polybrene for 3 hr with SL3-3 virus. Twelve or 36 hr after the virus had been washed out, various concentrations of suramin were added to the tissue culture medium, and the cells were grown for 10 days in suramin-containing medium. The supernatant was assayed subsequently for infectious virus particles and RT activity (Fig. 5). Suramin was able to decrease the titer of infectious virus, but the decrease was less pronounced when suramin was begun 36 hr after viral infection. The fact that suramin can still depress viral titers 36 hr after viral infection could be explained by its ability to prevent newly released viral particles from infecting neighboring cells, thereby limiting viral spread.

Effect of Suramin on Granulocyte and Macrophage Precursors. Bone marrow cells were isolated from the femurs and tibias of BALB/c mice and plated in the presence of various concentrations of suramin. Colony formation was not significantly suppressed at suramin concentrations up to 100 μg/ml, but at 300 μg/ml it was completely inhibited (data not shown).

***In Vivo* Testing of Suramin in Rauscher MuLV-Infected BALB/c Mice.** A total of 79 mice was treated either with virus alone, with suramin only, or with virus and suramin; 10 untreated mice were used as control (Table 1). A 35% reduction in virus-induced splenomegaly was seen at a dose of 40 mg/kg i.v. given twice weekly ($P < 0.0001$ by t test), whereas the dose of 10 mg/kg i.v. given by the same schedule failed to decrease the spleen weight. This is not surprising, as shown by the suramin levels that were measured in pooled plasma samples 24 hr after the last dose of suramin. A dose of 40 mg/kg twice weekly yielded suramin levels between 58 and 83 μg/ml, whereas 10 mg/kg only resulted in plasma levels of 12–14 μg/ml. The biological effectiveness of these suramin levels correlates well with the *in vitro* analysis discussed above. Since we have observed neither drug toxicity nor maximally effective plasma levels, the dose of suramin can be further escalated.

Pretreatment of the mice with suramin at 40 mg/kg 24 hr before administration of the virus, followed by the same dose given twice weekly, yielded a decrease of virally induced splenomegaly by 25% ($P = 0.00042$). This decrease was less than that observed at the same dose level but without pretreatment. Comparison of the two groups receiving virus and suramin at 40 mg/kg per dose showed that the mean spleen weight without pretreatment was less than that observed with pretreatment ($P = 0.044$, one-sided test). This statistically significant decrease in effectiveness of suramin, if it is given before viral infection, may be explained by side effects of suramin on the host. At relatively high doses, suramin has been noted to be lympholytic (17). The much greater antiviral effect of suramin may thus be somewhat diminished by immunosuppression. Also of note is the slight, but dose-dependent, development of splenomegaly in the mice treated with suramin only ($P < 0.001$, Jonckheere–Terpstra method).

DISCUSSION

The spreading epidemic of AIDS calls for therapeutic interventions. To date, no specific therapy is available for prevention or treatment of viremia. Development of inhibitors of viral RT should have high priority for the following reasons: (*i*) RT is a virally encoded enzyme, (*ii*) normal host cells do not have RT activity, (*iii*) without functioning RT, viral infection cannot take place, and the viral life cycle is interrupted (31).

Pharmacological agents capable of inhibiting viral RT should have a high therapeutic index, and they should be designed to spare the granulocyte function as much as possible. Suramin, a competitive inhibitor of several retroviral RTs (18), seems to fulfill these criteria. In our murine systems, infection in the presence of suramin yielded viral titers several logarithms lower than control levels. A lesser degree of suppression of viral titers occurred if suramin was started only after infection; suramin was thus still able to

Table 1. *In vivo* testing of suramin in BALB/c mice

Suramin pretreatment, mg/kg	Suramin treatment,* mg/kg	Virus	Drug deaths, dead/total	Suramin plasma level, μg/ml	Spleen weight, g	% inhibition of splenomegaly	Significance, *P*
None	None	−	0/10	0	0.106 ± 0.016		
None	None	+	0/19	0	1.625 ± 0.391		
10	10	−	0/10	14.2	0.117 ± 0.013		
10	10	+	0/10	11.8	1.691 ± 0.056	0	0.62†
40	40	−	0/10	54.4	0.135 ± 0.016		
40	40	+	0/10	69.5	1.225 ± 0.153	25	0.00042
None	40	−	0/10	83.1	0.128 ± 0.023		
None	40	+	0/10	58.3	1.056 ± 0.232	35	<0.0001

*Suramin treatments: twice weekly for 6 doses.
†Not significant.

decrease viral amplification and spread throughout the cultures. Whether inhibition of reverse transcription is the sole mechanism of suramin's antiretroviral activity is an open question. The highly negatively charged suramin is known to bind to many proteins and to inhibit a large number of enzymes (17). It is quite possible that suramin has a wide range of antiretroviral effects and that it could interfere with crucial steps in the viral life cycle other than reverse transcription, such as binding of the virus to receptors on the cell membrane. Most important, though, our results show that suppression of viral titers depends on the continued presence of the drug. Suramin acts as a virustatic but not as a virucidal drug. Should it find clinical application, we predict that patients will need to have continued therapeutic drug levels in order to derive protection. This may not be feasible, since albuminuria and renal damage have been reported to result from exposure to the drug (17). Suramin therefore may have to be combined with other effective antiretroviral agents, or it may have to be chemically modified. In either case, effective screening procedures are required to test efficacy and toxicity.

The murine viral system described seems ideally suited as a model system. Candidate drugs or drug combinations can be tested for their ability to suppress plaque formation in the XC plaque assay (26) by using the thymotropic SL3-3 virus and Rauscher MuLV. Inhibition of infection of cultured murine T cells by SL3-3 virus is analyzed in parallel. Cytotoxicity studies should include analysis of drug effects on granulocyte/macrophage colony-forming units. Drugs or drug combinations with promising therapeutic indices can then be analyzed for their ability to prevent HTLV-III-induced cytopathic effects *in vitro* (19).

Candidate drugs passing these first series of tests successfully are selected for *in vivo* testing by using the Rauscher MuLV/BALB/c system for expeditious determination of effective antiretroviral dosages. Because of the rapid onset of Rauscher MuLV-induced erythroleukemia, answers can be obtained within 3 weeks. The ability of the same drug dosage to suppress infection of T cells *in vivo* can be studied next, by using strains of mice susceptible to SL3-3 virus. The animals can be examined for SL3-3 viral titers as well as for development of thymic lymphoma and leukemia. Drug levels in the mice are determined to facilitate subsequent clinical trials of promising therapeutic agents.

The major advantage of such a system is the rapidity with which answers can be obtained. Unlike HTLV-III, which has an incubation time of 2 or even 5 years, SL3-3 induces disease in about 60–90 days. The continuing rapid spread of HTLV-III infection should make the drug screening system we have described highly attractive.

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