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Inhibition of HIV replication in lymphocyte cultures of virus-positive subjects in the presence of Sho-saiko-to, an oriental plant extract

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Summary

An oriental remedy, Sho-saiko-to (SST) consisting of a mixture of aqueous extracts from seven different plants and whose most active component is the chemically defined compound baicalein was tested for its ability to inhibit the production of the human immunodeficiency virus (HIV). The testing was done with cultures of human lymphocytes obtained from HIV-positive asymptomatic subjects and patients with ARC or AIDS. The replication of the virus was monitored by quantitative assay of the reverse transcriptase (RT) activity and of the synthesis of antigen p24. The lymphocyte cultures (LC) were maintained in the absence and in the presence of 25, 50 or 100 μg/ml of SST, and monitored for up to 5 weeks.

The results showed that in LC from asymptomatic subjects RT activity and synthesis of p24 was completely inhibited by low concentrations of SST. High concentrations of SST inhibited virus replication in 80% of LC from ARC patients, but were completely ineffective in LC from AIDS patients. It was observed that the RT activity was more sensitive to inhibition by SST than the synthesis of p24, and that the antiviral effect was dependent on the virus load of the LC.

HIV; Oriental plant extract; Inhibition; Reverse transcriptase; p24

Introduction

The search for treatment of acquired immunodeficiency syndrome (AIDS) has been directed not only towards well-characterized compounds with an expected

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mode of action, but also towards the domain of traditional medicine in which the use of natural substances, mainly from plants, is based on a long practical experience (Becker, 1980). A number of natural products have been tested for their ability to inhibit the replication of HIV and other retroviruses and several have been proven to be effective. Among these products are castanospermine which prevents syncytium formation and glycoprotein processing (Gruters et al., 1987; Sunkara et al., 1987; Walker et al., 1987), hypericin and pseudohypericin which may interfere with the processing of virus precursor polyproteins (Meruelo et al., 1988; Lavie et al., 1989), as well as glycyrrhizin (Ito et al., 1987), avarol and avarone (Sarin et al., 1987), extracts of *Viola yedoensis* (Chang and Yeong, 1988; Ngan et al., 1988) and gossypol extracted from cotton seed (Lin et al., 1989).

Recently, an oriental remedy called Sho-Saiko-to (SST), a mixture of aqueous extracts from the dried granules of *Bupleuri radix, Pinelliae tuber, Scutelarie radix, Ziziphy fructus, Ginseng radix, Glycyrrhizae radix* and *Zingeberis rhizoma* was tested for inhibition of the human immunodeficiency virus (HIV). It has been shown that SST at a concentration of 100 μg/ml inhibited about 50% of the reverse transcriptase (RT) activity of HIV (Ono et al., 1990). It appears that the most active component of SST is the extract of *Scutelariae radix*, which at a concentration of 50 μg/ml completely inhibited RT activity. The active component in the extract was identified as 5,6,7-trihydroxyflavone (baicalein) (Ono et al., 1989). The RT activity of murine leukemia virus and of HIV was reduced by more that 90% in the presence of 2 μg/ml Baicalein (Ono et al., 1989). Recently it has been shown that SST differentially inhibited the activities of RT and human cellular DNA polymerases α and β: retrovirus RT was at least five times more sensitive to SST than cellular DNA polymerases (Ono et al., 1990).

Unlike the above study we tested the inhibitory activity of SST not on cell-free virus preparations, but on lymphocyte cultures (LC) obtained from subjects of the various groups of the study population. In addition, the activity of SST was evaluated not only by RT activity released in the LC supernatant, but also by determining the presence of HIV p24 antigen released in the same supernatant. We will show that the extent of HIV inhibition by SST depends on the assay used for the evaluation of antiviral activity. Furthermore, the results will indicate that the ability of SST to inhibit the expression of HIV enzymes and antigens is dependent on the clinical stages of the disease.

Materials and Methods

Study population

Blood for lymphocyte cultures was obtained from 12 asymptomatic, HIV-positive homosexual men, 5 patients with ARC, and five AIDS patients. All subjects are part of a cohort which is under our clinical and laboratory observation since 1982. Prior to enrollment informed consent was obtained from all subjects in the study.

Lymphocyte cultures (LC)

Peripheral blood lymphocytes (PBL) were isolated from the interface after centrifugation of equal volumes of blood and Ficoll-Hypaque. The cells were washed twice in Hank's solution without calcium, and established in cultures containing 10^6 cells per ml of growth medium (RPMI with 10% fetal bovine serum and 10% interleukin-2). PBLs from the study subjects were mixed in a ratio of 1:1 with PBLs from a healthy HIV-negative donor. Donor cells isolated in the same way were stimulated previously for 3 to 5 days with phytohemagglutinin (Bacto) diluted 1:200. The cell mixtures were divided in four equal aliquots of 10^7 cells in 10 ml. One of the four samples served as a control, and the other three were supplemented with SST to the desired concentration. After every 3 to 4 days the medium was removed and fresh medium with corresponding concentrations of SST was added. Every 7 or 8 days, after replenishing the medium, the cultures were supplemented at a ratio of 1:1 with donor cells which had been maintained for not more than 12 days in culture. During the duration of the experiment two to three bleedings of the donor were necessary. Samples of culture supernatants (cell-free) were frozen at −70°C for RT assay and detection of p24 antigen. RT was assayed only in those samples in which a significant amount of antigen p24 was detected (100 pg/ml). The lymphocyte cultures from ARC and AIDS patients were maintained for up to 18 days, while those from asymptomatic subjects for up to 36 days.

Reverse transcriptase was assayed as previously described (Buimovici-Klein et al., 1986). *HIV p24 antigen* was detected and quantified with an ELISA commercial kit (Du Pont, Wilmington, Delaware).

Compound SST in soluble form was dissolved in phosphate-buffered saline and filter-sterilized. Final concentrations of 25, 50, and 100 μg/ml were used throughout the experiments. Preliminary tests on the toxicity of SST in normal and HIV-infected cells showed that a concentration of 200 μg/ml decreased the viability of cells by 20 to 25% as detected by Trypan blue exclusion. All three doses used in the experiments were non-toxic for fresh PBLs.

Results

Reverse transcriptase activity in lymphocyte cultures from HIV-positive asymptomatic subjects was completely suppressed in all subjects by SST concentrations of 50 or 100 μg/ml. Even a SST concentration of 25 μg/ml suppressed RT activity in 90% of the cultures. In the absence of SST all LC from asymptomatic individuals presented evidence of RT activity, though in one case this activity was observed only on day 36 of the lymphocyte culture (Fig. 1A).

RT activity was suppressed in 80% of the lymphocyte cultures from ARC patients by SST concentrations of 50 or 100 μg/ml, but was not affected by a drug concentration of 25 μg/ml. In the absence of SST all lymphocyte cultures presented evidence of RT activity by day 15 of cultivation. In the presence of 25 μg/ml SST all lymphocyte cultures became RT positive by day 19 of cultivation (Fig. 1B).

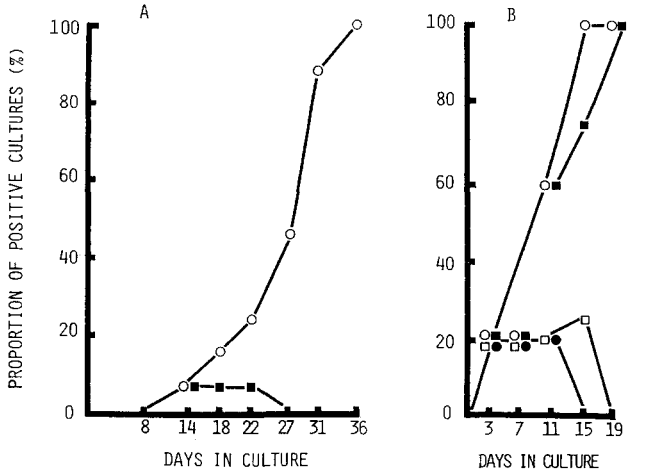

Fig. 1. Reverse transcriptase activity in the presence of SST in lymphocyte cultures from HIV antibody positive (A) asymptomatic subjects and (B) ARC patients: (○) no drug, (■) 25 μg/ml, (□) 50 μg/ml and (●) 100 μg/ml.

RT activity was not inhibited by any of the tested drug doses in lymphocyte cultures of AIDS patients. In the absence of SST all lymphocyte cultures became

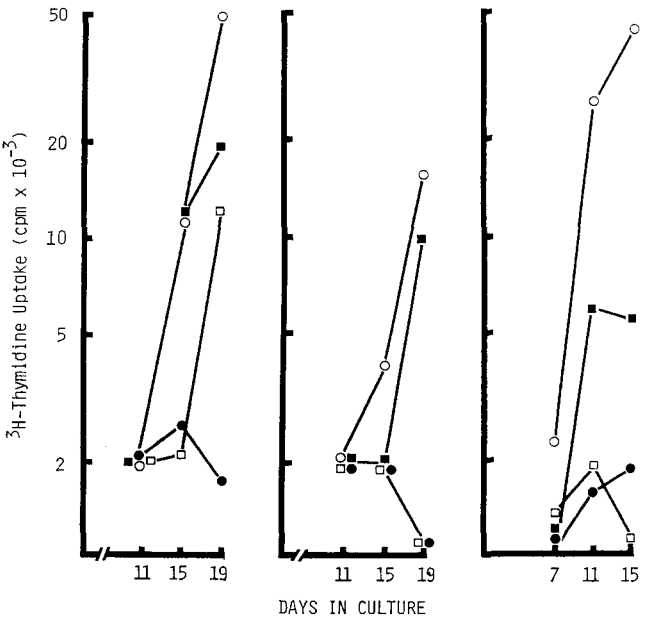

Fig. 2. Reverse transcriptase activity in the presence of SST in individual lymphocyte cultures from three typical ARC patients: (○) no drug, (■) 25 μg/ml, (□) 50 μg/ml, and (●) 100 μg/ml.

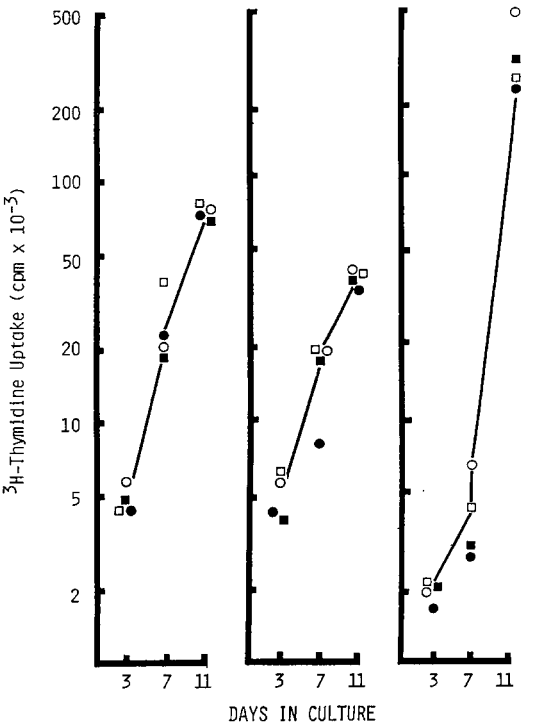

Fig. 3. Reverse transcriptase activity in the presence of SST in individual lymphocyte cultures from three typical AIDS patients: (○) no drug, (■) 25 μg/ml, (□) 50 μg/ml, and (●) 100 μg/ml.

RT positive between day 3 and 7 of cultivation, and in the presence of SST all cultures had evidence of RT activity by day 11 of cultivation.

The RT activity of individual lymphocyte cultures from ARC and AIDS patients in the presence of SST is shown in Figs. 2 and 3. It can be seen that in LC of three ARC patients peak activity of 10^4 to 5×10^4 counts per minute was attained after 19 days of cultivation (Fig. 2), whereas in LC from AIDS patients peak RT activity of up to 5×10^5 counts was observed only after 11 days of cultivation (Fig. 3). The higher number of counts attained earlier in AIDS than in ARC patients indicates the presence of a significantly heavier virus load.

Antigen p24 synthesis in LC from HIV-positive asymptomatic individuals was inhibited in over 90% of the cultures in the presence of SST at a concentration of 100 μg/ml. Concentrations of 25 or 50 μg/ml inhibited the synthesis of p24 antigen in 70 to 75% of the cultures. In the absence of SST all LC became positive by day 27 of cultivation (Fig. 4A).

The synthesis of p24 antigen was not inhibited in LC from patients with ARC. All untreated cultures, as well as those treated with SST at concentrations of 25 or 50 μg/ml, became p24 positive by day 11 of cultivation, whereas those treated with 100 μg/ml of SST became p24-positive by day 15 (Fig. 4B).

In LC from patients with AIDS none of the tested SST concentrations prevented

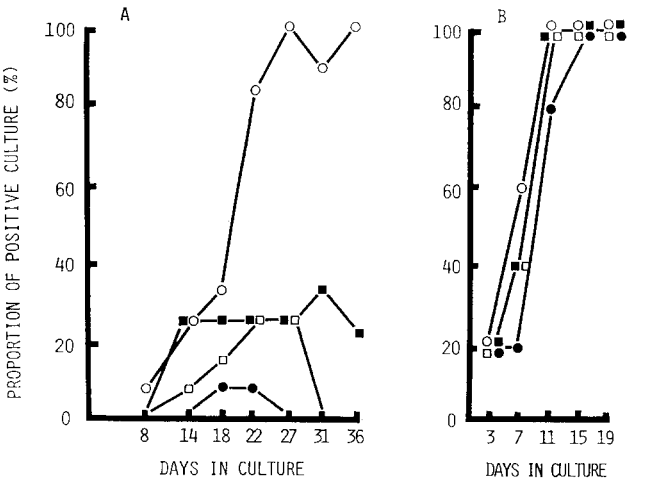

Fig. 4. HIV p24 antigen in the presence of SST in lymphocyte cultures from HIV antibody positive (A) asymptomatic subjects and (B) ARC patients: (○) no drug, (■) 25 μg/ml, (□) 50 μg/ml, and (●) 100 μg/ml.

the synthesis of p24 antigen. All SST-treated and untreated LC from AIDS patients became p24-positive by day 3 of cultivation.

Discussion

The results indicate that SST can prevent HIV production as measured by RT and p24 antigen released into the supernatants of LC established from blood samples of HIV-positive asymptomatic subjects and patients with ARC, but not in LC originating from AIDS patients. The inhibitory effect of SST showed a dose-dependent and disease stage-dependent activity: low doses of SST prevented virus production in LC of asymptomatic subjects, medium and high doses were effective in LC of patients with ARC, while in LC from patients with AIDS none of the tested doses of SST could prevent virus synthesis. This outcome suggests that the inhibitory activity of SST depends on the virus-load carried by the various categories of HIV-positive subjects. Previous studies have demonstrated an inhibitory effect of baicalein present in SST on the RT activity in vitro (Ono et al., 1989, 1990). It is therefore possible that the inhibition of viral production we have observed results from a direct effect of SST on RT activity within the infected cells.

It is likely that the mode of inhibition of RT by SST is similar to that exerted by two components of *Camellia sinensis* (tea plant). It was shown that their mode of inhibition of RT was competitive with respect to the template-primer whereas the mode of inhibition of RNA polymerase was competitive with respect to the nucleotide substrate (Nakane and Ono, 1990).

Our observations indicate that the selection of assays for the detection of HIV replication has an important role in evaluating the efficacy of antiviral compounds.

The results showed that when RT activity is used as an assay, SST in doses of 50 or 100 μg/ml is very effective in LC of asymptomatic persons. However, when p24 is used in the assay these doses are moderately effective only in asymptomatic subjects. This observation should be taken into account when the effectiveness of anti-HIV substances tested by different procedures is compared.

Similar observations have been made previously by other investigators (Gupta et al., 1987; Healey et al., 1987; Land et al., 1989). No satisfactory explanation has yet been provided for this observation. One reason could be the residual presence of drugs in the medium when RT activity is assayed. However, the dilution of test mixtures would generally reduce the actual concentration of drugs to levels at which they are ineffective. On the other hand, antigen p24 is unlikely to interact directly with SST, and therefore any residual amount of the antigen will be detected by the assay.

The inhibitory activity of antiviral drugs should logically be virus-load dependent. We selected groups of patients who provided the clinical specimens in which we were able to demonstrate the existence of the less explored relationship between drug dose and virus-load of patients. Indeed, it has been shown that virus titers in the blood of HIV-positive individuals is 10 to 100 times higher in patients with ARC or AIDS than in asymptomatic subjects (Ho et al., 1989; Coombs et al., 1989). Our quantitative assay of RT in lymphocyte cultures of healthy and diseased HIV-infected subjects confirm that the virus titers increase during the progression of the infection, and that the inhibition of virus production becomes more difficult to achieve.

The clinical potential of SST remains to be evaluated. Concentrations of 50 to 100 μg/ml in serum may not be too difficult to attain, but, until the compound is tested in an animal model, the relation between the effective in vitro and in vivo dose remains open to speculation.

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