A SUPPRESSOR LYMPHOKINE PRODUCED BY HUMAN T LEUKEMIA CELL LINES

Partial Characterization and Spectrum of Activity Against Normal and Malignant Hemopoietic Cells

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Several glycoproteins or polypeptides that are produced by T cells in culture (T lymphokines) and that inhibit cell proliferation in vitro have been identified and characterized. Two of these factors, immune interferon (IFN-γ) (1–3) and one member of the lymphotoxin (LT) family (4–6), have been purified to homogeneity, cloned, and sequenced (1–3, 7, 8). Several others are at different stages of characterization and, based on their most prominent biological activity, have been designated: inhibitor of DNA synthesis (IDS) (9–11); suppressor cell induction factor (SIF) (12, 13); macrophage migration inhibitory factor (MIF) (4, 14); soluble immune suppressor supernatants (SISS-T and SISS-B) (15, 16), which inhibit T cell proliferation or B cell Ig production; B cell growth inhibitory factor (BIF) (17); suppressor activating factor (SAF) (18, 19); soluble immune response suppressor (SIRS) (20–23); a factor that inhibits mouse bone marrow and leukemia cell proliferation (STIF) (24); and colony-inhibiting lymphokine (CIL), a factor that inhibits colony formation of human bone marrow progenitor cells (25). Several of these factors (LT, BIF, SISS-B, CIL) have been shown by column chromatography to have M₉ of 70,000–90,000, and their possible unique identity has been attributed either to their specific physicochemical characteristics (heat and pH sensitivity) or to their spectrum of target reactivity.

Part of the problem hampering the rapid purification and definitive characterization by protein and nucleic acid sequencing of these lymphokines lies in the fact that most are produced in small amounts by short term cultures of human PBL stimulated to proliferate by mitogens or alloantigens. Some factors, however, are abundantly elaborated by T cell lines: SAF is produced by a subclone of the 6-thioguanine (6TG)-resistant human leukemic T cell line CEM

†Abbreviations used in this paper: BIF, B cell growth inhibitory factor; BPA, burst-promoting activity; CIL, colony-inhibiting lymphokine; CSF, colony-stimulating factor; IDS, inhibitor of DNA synthesis; LT, lymphotoxin; SAF, suppressor-activating factor; SIF, suppressor cell induction factor; SIRS, soluble immune-response suppressor; SISS, soluble immune suppressor supernatants; SPN, supernatant; 6TG, 6-thioguanine; TLI., T leukemia-derived suppressor lymphokine.

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(18), and CII was identified in the supernatant of a putative T cell hybridoma between the 6TG-resistant T leukemia cell line Jurkat and mitogen-stimulated human lymphocytes (25). However, firm evidence that the producing clone (MT1) is truly a hybrid and not just a subclone of Jurkat cells is lacking because (a) only HLA surface markers of the Jurkat cell line could be detected (25), and (b) analysis of the clone with a polymorphic probe (Pow101) (D14S1) (26) showed a restriction pattern type of the parental Jurkat cells and not of the lymphocytes used as fusion partners (our unpublished observations).

We report here that all the T leukemia cell lines tested constitutively produce high concentrations of a factor that displays potent antiproliferative effects against a broad spectrum of normal and malignant cells of hemopoietic origin. The biological effects of this T leukemia–derived suppressor lymphokine (TLSL), as well as its physicochemical and M, characteristics are presented.

Materials and Methods

Cell Lines. Seven established T lymphoblastoid cell lines were examined for spontaneous production of factors with antiproliferative activity. They included Jurkat (27), CCRF/HSBz (28), CCRF/CEM (29), MOLT-4 (30), JM (31), HPB-ALL (32), and HUT-78 (33). All of these lines originated from patients with acute T cell leukemia, except HUT-78, which was derived from a patient with chronic T cell leukemia. With the exception of HPB-ALL cells, which showed a low level of micoplasma contamination, all of the lymphokine producer cell lines were micoplasma-free, as determined by the methods of growth in agar (34) and the Hoechst DNA stain (35). Target hemopoietic cell lines of non-T origin included: three Burkitt lymphoma–derived B lymphoblastoid lines (Raji, Daudi, and BL2) four myelogenous leukemia cell lines (HL60, ML3, KG1, and K562), the histiocytic lymphoma line U937, and the BV-173 line established from a patient with chronic myeloid leukemia (CML) in lymphoid blastic crisis (for review see 36).

All of the cell lines described above were grown at 37°C in a 5% CO2 atmosphere in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Flow Laboratories, Rockville, MD), glutamine, and antibiotics.

Lymphocyte Cultures. Peripheral blood mononuclear cells from healthy donors were separated by centrifugation on a Ficoll-Hypaque gradient and incubated in the presence of 1% PHA (Burroughs Wellcome, Greenville, NC) or 0.5% PWM (Gibco, Grand Island, NY). MLC were performed by mixing freshly separated PBL with allogeneic γ-irradiated (4,500 rad) PBL at a responder/stimulator ratio of 1:1 or 1:2 in RPMI 1640 medium containing 15% heat-inactivated human AB serum. MLC and mitogen-stimulated PBL were used as targets to investigate the susceptibility of in vitro–activated hemopoietic cells to the T cell leukemia–derived suppressor lymphokine. In some of the assays, purified T cells were stimulated with PHA or in MLC and used as targets as compared to unfractonated PBL; purified T lymphocytes were obtained after depletion of monocytes, B, and null cells by adherence to plastic, phagocytosis of carbonyl iron particles, adherence to nylon wool columns, and depletion of non-E rosetting cells, as described elsewhere (37).

Myeloid and Erythroid Progenitor Cells. Bone marrow and peripheral blood mononuclear cells from healthy donors were separated on Ficoll/Hypaque gradients. Both samples were depleted of adherent cells by two incubations of 60 min each in plastic flasks in RPMI medium containing 20% FBS at a concentration of 5–10 × 10⁶ cells/ml. Peripheral blood CFU-GM and BFU-E were further depleted of monocytes and B cells by passage on nylon wool columns, and were depleted of T lymphocytes by elimination of E-rosetting cells as described previously (37). The rosetting technique with neuraminidase-treated sheep E was repeated twice to obtain ≥99% depletion of T cells. Phagocytic cells were then removed by carbonyl iron treatment, as described (37).

Production and Fractionation of TLSL. 3–4 d after their passage, exponentially growing T leukemia cell lines were centrifuged at 3,500 rpm for 20 min at 4°C, and the cell-free
supernatants (SPN) were harvested. For gel filtration, cells were grown in 500 ml of tissue culture medium to a density of 1-2 \times 10^6 cells/ml, transferred to either 1 liter of serum-free synthetic medium (38) or to RPM1 medium containing 5% FBS, and incubated at 37°C in a CO2-enriched humidified atmosphere for either 5 or 2 d, respectively. Cell-free crude SPN were harvested, solid ammonium sulfate was added to achieve 85% saturation, and the solution was equilibrated for 20 min before centrifugation at 13,200 g for 20 min at 4°C. The resultant precipitate was resuspended in PBS placed in dialysis tubing (Spectrapor; Spectrum Medical Industries, Los Angeles, CA) and dialyzed extensively at 4°C against PBS or PBS plus 0.5 M NaCl, or PBS plus 0.02% Tween-20 (Bio-Rad Laboratories, Richmond, CA). The dialyzed material was centrifuged at 13,200 g for 20 min at 4°C to remove large aggregates. A 4-ml aliquot was placed on a Sephacryl S-300 column (1.5 x 50 cm; Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS or PBS plus 0.5 M NaCl, or PBS plus 0.02% Tween-20, and 3-ml fractions were collected at a flow rate of 10 ml/h at 22°C. Fractions were tested for antiproliferative activity as described below.

**Proliferation Assay.** The antiproliferative activity present in the crude and partially purified SPN was measured against a variety of target cells. Hemopoietic tumor cell lines were seeded in triplicate wells of microtiter plates at 2-4 x 10^5 cells in 100 μl/well in tissue culture medium, and 100 μl/well of SPN at various concentrations was added. After a 5-d incubation at 37°C (conditions predetermined to give maximal inhibition of proliferation without significant loss of cell viability) 2 μCi [^3H]TdR (2 Ci/mmol) per well was added. The cultures were harvested 7 h later on fiberglass filters, and isotope incorporation was measured by scintillation counting. Alternatively, cells were seeded in Linbro plates at 2-4 x 10^5 cells in 2 ml/well in the presence of several SPN concentrations; on day 5, the cells were counted and viability was monitored by Erythrosin B dye exclusion test. The cells were washed and transferred to microplates at 2 x 10^5 viable cells/well and [^3H]TdR was added for 7 h to triplicate cultures. When mitogen- or alloantigen-stimulated lymphocytes were used as targets, these were plated at a concentration of 1-2 x 10^5 cells/well in microtiter plates and incubated with lymphokine-containing SPN for 3-4 d (in the case of PHA-stimulated cells) or for 6 d (in the case of PWM- or MLC-stimulated cells) before addition of [^3H]TdR.

**Clonogenic Assay.** Tumor cell lines were incubated at 5 x 10^5 cells/well of Linbro plates in 2 ml medium containing 10-fold dilutions of T cell leukemia-derived SPN. On day 4, cells were monitored for viability and cell concentration. Cells were then seeded in plastic Petri dishes at 2 x 10^5 in 0.9% methylcellulose (Dow Chemical Co. Midland, MI) and complete medium supplemented with 10-fold serial dilutions of crude SPN. The number of viable colonies was determined after 14-d incubation at 37°C, and was compared to the growth of suspension cultures incubated in microplates for the same length of time.

**Culture Assay for CFU-GM and BFU-E.** Bone marrow and peripheral blood CFU-GM were cultured, as previously described (25), in 35-mm Petri dishes in Iscove's modified Dulbecco's medium containing 20% FBS, 0.3% agar, 10% Giant Cell Tumor cell line (GCT)-conditioned medium (Gibco) as a source of colony stimulating factor (CSF), and different concentrations of T leukemia-derived SPN. Colony growth of late (less immature) and early (more immature) CFU-GM was monitored after 7 and 14 d of incubation, respectively, at 37°C in a 5% CO2 atmosphere.

BFU-E were cultured, as previously described (25), in Iscove's modified Dulbecco's medium containing 0.8% methylcellulose, 30% FBS, 7% lymphocyte-conditioned medium (obtained from human PBL cultured for 7 d in the presence of PHA) as a source of burst-promoting activity (BPA), 10^-4 M β-mercaptoethanol, 1.5 U/ml sheep erythropoietin step 3 (Connaught Laboratories, Ltd., Ontario, Canada), and various concentrations of T leukemia-derived SPN. BFU-E-derived colonies were scored after 14 d of incubation.

**IFN Assay.** Antiviral titers were measured in the crude SPN and in the fractionated material by inhibition of the cytopathic effect of vesicular stomatitis virus on a variety of target fibroblast lines, including bovine brain (CB1), human fetal skin (FS-1, Ag-10, and Ag-1603) and human foreskin (HF) fibroblasts. The assay included the National Institutes
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Interleukin 2 (IL-2) Assay. The levels of IL-2 present in the conditioned medium of T leukemia cell lines were measured by testing the ability of cell-free SPN to support the growth of IL-2-dependent human T cell clones, as described elsewhere (40).

DNA Cell Cycle Analysis. Cellular DNA of tumor cell lines cultured for 1–7 d in medium alone or in the presence of TLSL was examined by staining the cells with propidium iodide (41). Cells were seeded in T25 plastic flasks at 2 x 10^5 cells/ml. At various intervals after addition of 10% crude SPN, cells were plated as 5.0 x 10^5 cells/well, fixed with cold 70% ethanol for 10 min, washed, and resuspended in PBS containing 0.2 μg/ml propidium iodide and 62 μg/ml RNase (Sigma Chemical Co., St. Louis, MO). Cells were then examined by flow cytometry.

Results

Inhibition of Proliferation of Leukemia and Lymphoma Cell Lines. The presence of antiproliferative activity in the T cell leukemia-derived conditioned medium was measured by monitoring daily the cell number, viability, and [3H]TdR incorporation of leukemia/lymphoma target cell lines incubated in the presence of crude SPN. Fig. 1 depicts a representative experiment in which Daudi, sensitive to all of the SPN, and BV-173, not inhibited by the Jurkat SPN (see Table I), were used. In the first 5 d, the viability and concentration of Daudi cells incubated in the presence of either Jurkat or CCRF/CEM SPN were very similar to those in control cultures. In contrast, [3H]TdR incorporation was already reduced on the third day, especially in the cultures incubated with the CCRF/CEM SPN. By day 7, the treated cells displayed very low viability and ability to incorporate [3H]TdR, and by day 9, they were all dead (Fig. 1). BV-173 cells incubated with CCRF/CEM SPN were still 90% viable on day 5, but became much less viable and able to proliferate during the following days. In contrast, the viability, concentration, and [3H]TdR incorporation of BV-173 cells incubated in the presence of the Jurkat SPN were identical to those of the control cultures throughout the 9-d period examined (Fig. 1). All of these observations demonstrate that the inhibitory activity of TLSL is cytostatic rather than cytotoxic.

Because isotope incorporation was the most sensitive indicator of inhibition, subsequent experiments were performed by seeding the experimental and control cells directly in microplates and comparing [3H]TdR incorporation on day 5. Table I shows the spectrum of reactivity of SPN from seven human T leukemia cell lines against a panel of leukemia/lymphoma target cell lines. The SPN from Jurkat cells strongly inhibited [3H]TdR uptake by two Burkitt lymphoma cell lines (Raji and Daudi), and slightly inhibited the growth of another Burkitt lymphoma (BL2) and of the myeloblastic leukemia KG1 cells; the other SPN drastically inhibited [3H]TdR incorporation of all target cells tested (Table I). The myelogenous leukemia cell lines HL60, ML3, and K562 were slightly less susceptible than others to the inhibitory activity of most SPN. The production of TLSL was restricted to leukemia cell lines of T cell origin: crude SPN obtained from Raji and U937 cells (Table I) and from seven other cell lines of non-T origin (Daudi, BL2, K562, HL60, KG1, BV-173, ML3) (data not shown) did not inhibit the growth of any hemopoietic cell line tested.

Titration experiments against Raji cells (one of the most sensitive targets)
Figure 1. Kinetics of inhibition of proliferation. Daudi and BV-173 cells were suspended at 10⁶ and 5 × 10⁴ cells/ml respectively, and incubated at 37°C in five separate tissue culture flasks in medium containing 10% crude SPN from Jurkat and CCRF/CEM cell lines. Control cultures were incubated in medium with no inhibitory factors. On alternate days, aliquots of cells were removed from each flask and their concentration and viability were monitored. The cell concentration in each sample was adjusted to 10⁶ cells/ml and [3H]TdR was added for 7 h to triplicate cultures containing 2 × 10⁵ cells/well. Neither fresh medium nor additional T leukemia–derived SPN was added during the entire 9-d incubation period.

Table I

Antiproliferative Activity of T Cell Leukemia–derived SPN on Hemopoietic Tumor Lines of Non-T Origin

| SPN            | Raji | Daudi | HTL2 | KG1 | HL60 | ML3 | U937 | K562 | BV-173 |
|----------------|------|-------|------|-----|------|-----|------|------|--------|
| Jurkat         | 89.2±7.2 | 94.0±4.2 | 56.1±4.2 | 16.0±4.3 | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0  |
| CCRF/HSB7     | 95.6±4.4 | 90.1±6.4 | 78.0±8.5 | 86.0±0.0 | 63.0±8.5 | 78.5±6.4 | 90.5±3.5 | 75.3±3.8 | 95.7±4.2 |
| JM            | 97.0±5.2 | 81.3±5.7 | 59.1±3.7 | 89.0±3.2 | 66.3±5.7 | 84.5±2.1 | 88.4±1.7 | 79.4±1.8 | 84.5±5.2 |
| HPB-ALL       | 96.2±5.6 | 96.4±1.9 | 92.5±5.6 | 92.7±2.1 | 87.8±5.6 | 90.4±3.8 | 86.3±6.4 | 75.2±2.1 | 89.7±3.5 |
| MOLT-4         | 96.8±3.3 | 85.8±8.8 | 91.0±6.8 | 90.6±5.0 | 64.0±5.9 | 79.2±7.5 | 90.3±3.4 | 88.0±1.4 | 88.7±6.7 |
| MOLT-8         | 97.8±1.5 | 94.3±4.1 | 96.9±2.5 | 95.3±4.0 | 92.5±7.1 | 90.5±4.1 | 96.6±3.5 | 90.7±2.7 | 94.5±3.3 |
| CCRF/CEM      | 96.4±1.5 | 97.2±1.5 | 95.7±3.8 | 91.0±2.6 | 89.0±5.3 | 93.5±7.4 | 88.5±0.7 | 90.0±5.1 | 96.5±2.7 |
| Raji           | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0  |
| U937          | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0 | ±1.0  |

*Values represent the percent inhibition of proliferation observed in 4–9 experiments (mean ± SD).
revealed that the SPN from CCRF/CEM, MOLT-4, and HUT-78 cells were the most inhibitory, being able to inhibit at very high dilutions ($10^{-9}-10^{-11}$) (Fig. 2); those derived from HPB-ALL, JM, and CCRF/HSB2 cells had a strong inhibitory activity (>80%) up to dilutions of $10^{-5}$, and the Jurkat SPN was the least active of all, being able to inhibit only at dilutions lower than $10^{-5}$. When the less susceptible K562 target cells were used, similar data were obtained, but the titer of each SPN was at least 100 times lower, and the Jurkat SPN was not active (Fig. 2).

Clonogenic assays were performed to determine whether TLSL could abrogate the ability of malignant targets to grow as clones in semisolid medium. After a 14-d incubation in methylcellulose, control cultures of Raji and Daudi cells were able to produce hundreds of large colonies containing viable cells. In contrast, the cultures incubated in the presence of 1:10 dilution of either CCRF/CEM or HUT-78 SPN formed few (≤10) small clumps containing 5–10 degenerated cells. The number and size of clumps or colonies became larger with increasing SPN dilutions, but the cells were mostly dead; at the highest dilutions used ($10^{-9}-10^{-11}$) the number of colonies detected and their viability was ~50% that of the control cultures. The reduction in clonogenic efficiency induced by the SPN at any dilution tested paralleled the inhibition in $[^3H]$TdR incorporation observed in the cells incubated in microplates for the same length of time. These data indicate that the clonability of tumor cell lines in semisolid cultures is not affected during the first few days of incubation with TLSL. Later on, however, cell replication is arrested and the elements present in each colony undergo degenerative processes.

Inhibition of proliferation was not detected in T cell lines during a 5-d incubation with the T leukemia-derived SPN, with two exceptions: the Jurkat cell line, which was susceptible to every SPN, and the CCRF/HSB2 cell line, in which proliferation was partially inhibited by SPN from CCRF/CEM, HUT-78, and MOLT-4 cells (Table II). Thus, the low-producer T cell line Jurkat was the most susceptible to the inhibitory activity, the intermediate-producer T cell line
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Table II

Antiproliferative Activity of T Cell Leukemia-derived SPN on Hemopoietic Tumor Cell Lines of T Cell Origin

| SPN       | Jurkat | CCRF/HSB2 | JM, HPB-ALL, MOLT-4, HUT-78, CCRF/CEM |
|-----------|--------|-----------|-------------------------------------|
| Jurkat    | <1.0*  | ≤1.0      | ≤1.0                                |
| CCRF/HSB2 | 55.5 ± 10.5 | ≤1.0 | ≤1.0                                |
| JM        | 55.1 ± 4.7  | ≤1.0      | ≤1.0                                |
| HPB-ALL   | 38.4 ± 2.9   | ≤1.0      | ≤1.0                                |
| MOLT-4    | 68.5 ± 4.9   | 24.0 ± 2.2 | ≤1.0                                |
| HUT-78    | 81.3 ± 8.8   | 46.2 ± 11.3 | ≤1.0                                |
| CCRF/CEM  | 80.3 ± 8.6   | 74.6 ± 3.2 | ≤1.0                                |

T cell lines (2-4 × 10^5 cells/well) were incubated in the presence of T leukemia-derived crude SPN (diluted 1:10) for 5 d before addition of [3H]TdR. Values represent percent inhibition of [3H]TdR incorporation (mean ± SD) observed in three or four experiments.

CCRF/HSB2 was partially sensitive, and the high-producer lines were not affected at all. In no instance did the SPN inhibit the proliferation of the cells from which they were derived (Table II). However, partially purified and more concentrated material derived from the conditioned medium of the Jurkat cell line inhibited the growth of Jurkat cells, as well as of the other T cell lines that were insensitive to the crude preparation (data not shown). Autologous pairs of Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines and IL-2-dependent T cell clones, all established in our laboratory from the blood of healthy donors, were used as target cells in some experiments: the T cell clones were only slightly sensitive to the inhibitory activity of the crude SPN, whereas their B cell counterparts were highly susceptible (data not shown). Under the same experimental conditions described above, neither HeLa cells nor mouse L fibroblasts were affected by the presence of Jurkat SPN at any dilution tested (10^-1–10^-9). The SPN from CCRF/CEM cells was not effective against mouse L cells but inhibited the proliferation of HeLa cells up to the 10^-5 dilution; by day 7, these cells were all detached from the plastic and had a viability 60–70% lower than control HeLa cells.

To determine whether the continuous presence of TLSL was required for the inhibitory effect, proliferation was compared in Raji and BV-173 cells that had been either cultured for 4–5 d in the presence of crude SPN or treated with the same SPN for shorter time periods. Results indicated that a 1-h incubation with TLSL was sufficient to result in significant inhibition of proliferation (up to 75% in the case of Raji and up to 69% in the case of BV-173 cells) (Table III). The levels of inhibition observed after 3 h, 7 h, or after overnight treatment were similar, and approached those observed in cultures continuously incubated with the SPN. Thus, reduced proliferative activity in target cells is observed even after a very brief exposure to TLSL, although its continuous presence results in
TABLE III
Kinetics of Inhibition of Proliferation of Tumor Cells Incubated with T Leukemia-derived SPN
For Various Time Periods

| SPN          | Raji cells plus SPN for: | BV-173 cells plus SPN for: |
|--------------|--------------------------|---------------------------|
|              | 1 h 3–16 h 5 d           | 1 h 3–16 h 4 d            |
| Medium       | 87,417*                  | 12,356                    |
| Jurkat       | 34,506 (61)              | 11,905 (4)                |
| CCRF/HSB2    | 42,907 (51)              | 10,437 (16)               |
| JM           | 36,724 (58)              | NT                       |
| HPB-ALL      | 28,411 (68)              | 3,891 (69)                |
| MOLT-4       | 25,743 (71)              | NT                       |
| HUT-78       | 21,919 (75)              | NT                       |
| CCRF/CEM     | 26,776 (70)              | 5,946 (52)                |

Raji and BV-173 cells were incubated at 37°C in 24-well Linbro plates for 1, 5, 7, and 16 h in the presence or absence of crude SPN (1:10 dilution); the cells were washed four or five times after each interval, and incubated in microplates (2 × 10³ cells/well in triplicate) for a total of 4–5 d in medium alone. Cultures that were incubated continuously in the presence of crude SPN over a period of 4–5 d were seeded directly in microplates (2 × 10³ cells/well) from day 0. Cells were harvested after a 7-h pulse with [³H]TdR.

* Data are given as mean cpm. The number in parentheses is the percent inhibition of proliferation.

maximal inhibition of proliferation. To investigate the possibility that the inhibitory factor partially or completely binds to the tumor target cells, cell-free SPN previously absorbed to Raji cells were tested against fresh Raji cultures at various dilutions. The adsorbed SPN were two to four orders of magnitude less active than the nonadsorbed one (Fig. 3), indicating that a large fraction of the inhibitory lymphokine was either inactivated by or adsorbed to the tumor target cells.

IL-2 and IFN assays. To investigate the possibility that either IL-2 or IFN were at least partly responsible for the inhibitory activity found in the T cell leukemia–derived SPN, IL-2 and IFN assays (using cells with various degrees of sensitivity to IFN-α; -β; or -γ) were performed. Results clearly demonstrated the absence of these lymphokines both in the crude SPN from each T leukemia cell
line, and in the purified fractions obtained from Jurkat and CCRF/CEM cells (data not shown).

**Cell Cycle Analysis.** Kinetics studies with propidium iodide staining and flow cytometric analysis indicated that upon incubation with TLSL, three tumor cell lines (HL60, Daudi, ML3) accumulated in the G1 phase of the cell cycle, whereas Raji cells were arrested in the S phase. A representative experiment is depicted in Fig. 4: after 7 d in culture, HL60 control cells (a) and HL60 cells incubated in the presence of Jurkat SPN (b) were 93% viable and could be found in each phase of the cell cycle (~50% in G1, 26% in S, and 20% in G2). In contrast, HL60 cells incubated with CCRF/CEM SPN (c) were <50% viable than control cultures, and the viable cells were not dividing, being preferentially accumulated in the G1 phase of the cell cycle (≥71% were in G1, 12% in S, and 12% in G2). Raji cells displayed a different cell cycle pattern: by day 7, control cultures (a) were still highly viable and distributed as 59% in G1, 16% in S, and 23% in G2. Raji cells incubated with Jurkat (b) or CCRF/CEM SPN (c) had a lower viability, and were 46–51% in G1, 33–36% in S, and 14–17% in G2. Thus, Raji cells incubated with TLSL tend to accumulate in the S phase of the cell cycle.

**FIGURE 4.** Cell cycle analysis. HL60 and Raji cells were incubated at 2 x 10⁵ cells/ml in T25 plastic flasks in medium alone (a) or in the presence of 10% crude SPN from Jurkat (b) or CCRF/CEM (c) cells. On day 7, cells were stained with propidium iodide and examined by flow cytometry. Regions 1, 2, and 3 correspond to the G1, S, and G2 phases of the cell cycle, respectively. The histogram shows fluorescence intensity (abscissa) and the cell number values (ordinate).
Inhibition of T Lymphocyte Activation. To determine whether TLSL affects the proliferation of hemopoietic target cells other than leukemia/lymphoma cell lines, we investigated the susceptibility to this factor of peripheral blood mononuclear cells induced to proliferate in vitro. Two systems of T cell activation were analyzed, one in response to alloantigens, and another in response to mitogens. While the SPN from Raji and Daudi cells had no antiproliferative effect, all of the T leukemia cell–derived SPN abrogated the response of lymphocytes to any stimulus (Table IV). The level of inhibition was the same whether TLSL was added at the start or 24–48 h after the initiation of the cultures (data not shown). Purified T cells stimulated by PHA or in MLC were inhibited to the same extent as unfractionated PBL by all SPN tested except for the Jurkat SPN (data not shown). Titration studies against PHA-stimulated lymphocytes revealed that the Jurkat and CCRF/HSB₂ SPN were the least active (Fig. 5), whereas SPN produced by CCRF/CEM and HUT-78 cell lines were again the strongest, resulting in >50% inhibition at 10⁻⁵. Overnight incubation of freshly obtained lymphocytes with T leukemia–derived SPN greatly diminished or abolished the proliferative response of these cells to PHA stimulation even after removal of the inhibitory SPN for the rest of the incubation period (Table V). Thus, short-term exposure of resting lymphocytes to TLSL severely affects their ability to respond to activation signals.

Inhibition of Growth of CFU-GM and BFU-E. The ability of TLSL to inhibit normal myeloid or erythroid colony formation was investigated. All of the crude SPN preparations completely abrogated the growth of early (day 14) CFU-GM from peripheral blood of healthy donors, when tested up to 10⁻⁴ dilution (Table VI). The SPN from Jurkat, HUT-78, and CCRF-CEM cell lines were the most active, being able to totally inhibit even at 10⁻⁵. Likewise, inhibition of growth

| SPN         | PHA⁺     | PWM⁻ | MLC⁻ |
|-------------|----------|------|------|
| Jurkat      | 83.2 ± 6.8¹ | 93.3 ± 4.0 | 98.2 ± 0.8 |
| CCRF/HSB₂   | 91.4 ± 3.1 | 92.5 ± 4.4 | 91.0 ± 6.5 |
| JM          | 91.0 ± 2.1 | 87.5 ± 2.1 | 92.7 ± 0.6 |
| HPB-ALL     | 91.2 ± 5.0 | 88.0 ± 2.8 | 91.0 ± 4.6 |
| MOLT-4      | 94.8 ± 2.5 | 95.2 ± 1.5 | 95.1 ± 2.9 |
| HUT-78      | 93.8 ± 2.8 | 90.1 ± 1.3 | 91.3 ± 6.3 |
| CCRF/CEM    | 95.0 ± 3.2 | 94.2 ± 3.5 | 97.6 ± 1.4 |
| Raji        | ≤1.0      | ≤1.0  | ≤1.0  |
| Daudi       | ≤1.0      | ≤1.0  | ≤1.0  |

¹ PHA-stimulated PBL or purified T lymphocytes were seeded at 1–2 × 10⁵ cells/well in the presence of crude SPN (1:10 dilution), and incubated for 3–4 d before addition of [³H]Tdr.

² PWM-stimulated PBL were plated as 1.5 × 10⁵ cells/well and incubated for 6 d.

³ MLC cultures were seeded as 1–1.5 × 10⁵ cells/well and incubated for 6 d.

¹ Percent inhibition of proliferation observed in 3–10 experiments (mean ± SD).
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**Figure 5.** Titration of the antiproliferative activity of TLSL against stimulated lymphocytes. PBL (2 x 10^5 cells/well in triplicate cultures) were incubated at 37°C in the presence of 1% PHA and 100-fold dilutions of crude SPN from Jurkat (○), HUT-78 (●), CCRF/HSB2 (□), MOLT-4 (■), HPB-ALL (△), CCRF/CEM (▲), and JM (◇) T leukemia cell lines. [3H]TdR was added for 7 h on day 4. The percent inhibition of isotope incorporation is based on 173,500 cpm of the control PHA-stimulated lymphocytes cultured without SPN.

**Table V**

| SPN          | [3H]TdR incorporation | Inhibition of proliferation |
|--------------|-----------------------|-----------------------------|
| Medium       | 97,439                | —                           |
| Raji         | 94,557                | 3                           |
| Jurkat       | 32,565                | 67                          |
| MOLT-4       | 4,072                 | 96                          |
| CCRF/CEM     | 1,864                 | 98                          |

PBL were incubated overnight at 37°C in medium containing crude SPN (1:10 dilution). Lymphocytes were then washed four or five times, PHA (1%) was added, and the cultures were incubated (10^5 cells/well) in the absence of inhibitory factors for 4 d before addition of [3H]TdR.

... of early CFU-GM from bone marrow could be seen with all of the SPN (except for JM) at least up to 10^-5 (Table VII). In contrast, bone marrow late CFU-GM were sensitive only to higher concentrations of the SPN (1:10 and in some cases 1:100 dilutions) (Table VIII). These results were identical to those previously reported for CIL (25).

Experiments in which peripheral blood CFU-GM were incubated for brief periods of time (1–12 h) with crude SPN at 1:10 and 1:100 dilutions, then washed free of SPN and incubated for 14 d more with CSF, indicated that 1-h exposure of early CFU-GM was sufficient to initiate inhibition at 1:10 dilution (Table IX). Nearly complete inhibition of growth was observed also at 1:100
TABLE VI
Titration of Colony-inhibiting Activity of T Leukemia-derived SPN
Against Peripheral Blood CFU-GM

| SPN         | Colonies developing at SPN dilutions of: |
|-------------|-----------------------------------------|
|             | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| Jurkat      | 0         | 3 ± 1      | 45 ± 15   |
| CCRF/HSB$_2$| 11 ± 1    | 63 ± 5     | NT*       |
| JM          | 16 ± 4    | 70 ± 2     | NT        |
| HPB-ALL     | 20 ± 6    | 44 ± 0     | 72 ± 8    |
| MOLT-4      | 5 ± 5     | 54 ± 4     | NT        |
| HUT-78      | 0         | 2 ± 0      | 65 ± 5    |
| CCRF/CEM    | 0         | 2 ± 2      | 71 ± 27   |

Peripheral blood mononuclear cells depleted of monocytes, B, and T lymphocytes were cultured in 35-mm Petri dishes in medium containing CSF and crude SPN at various dilutions. CFU-GM-derived colonies were counted on day 14 using an inverted microscope.

* Values represent the number of colonies scored in duplicate cultures (mean ± SEM). No colonies were detected in the cultures containing SPN at dilutions up to $10^{-3}$. The number of colonies in control cultures (grown in the absence of SPN) was $80 ± 8$ per $10^5$ mononuclear cells plated.

† NT, not tested.

TABLE VII
Titration of Colony-inhibiting Activity of T Leukemia-derived SPN
Against Bone Marrow Early CFU-GM

| SPN         | Colony growth at SPN dilutions of: |
|-------------|-----------------------------------|
|             | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ |
| Jurkat      | 0         | 6 ± 4     | 8 ± 3     | 20 ± 2    | 38 ± 8    |
| CCRF/HSB$_2$| 0         | 4 ± 1     | NT*       | 20 ± 5    | 36 ± 14   |
| JM          | 3 ± 2     | 20 ± 6    | NT        | 103 ± 20  | NT        |
| HPB-ALL     | 9 ± 2     | 2 ± 2     | NT        | 34 ± 11   | 37 ± 7    |
| MOLT-4      | 0         | 5 ± 2     | NT        | 24 ± 9    | 49 ± 4    |
| HUT-78      | 0         | 2 ± 2     | NT        | 25 ± 11   | 37 ± 6    |
| CCRF/CEM    | 0         | 3 ± 3     | 7 ± 2     | 30 ± 4    | 26 ± 8    |

Bone marrow mononuclear cells depleted of adherent cells were cultured in 35-mm Petri dishes in medium containing crude SPN at various dilutions. Early CFU-GM-derived colonies were counted on day 14.

* Values represent the percentage of colony growth (mean ± SEM) in the experimental cultures as compared to control cultures incubated in the absence of SPN. The number of colonies in control cultures were 123 ± 25 and 97 ± 8 per $10^5$ mononuclear cells in two experiments, with bone marrow samples from two different donors.

† NT, not tested.

dilution when CFU-GM were exposed to the inhibitory factor for 12 h (Table IX). These results suggest that TLSL binds to the target cells with a specific receptor and, in a fashion similar to other lymphokines, initiates a series of intracellular events that result in inhibition of proliferation.

The conditioned medium from the T leukemia cell lines also contained inhibitory activity against the growth of peripheral blood BFU-E. Titration
TABLE VIII
Titration of Colony-inhibiting Activity of T Leukemia-derived SPN Against Bone Marrow Late CFU-GM

| SPN            | Colony growth at SPN dilutions of: |
|----------------|----------------------------------|
|                | 10^{-1} | 10^{-2} | 10^{-3} |
| Jurkat         | 0*      | 3 ± 1   | 95 ± 6  |
| CCRF/HSB2      | 0       | 70 ± 6  | 99 ± 8  |
| JM             | 1 ± 2   | 105 ± 12| NT*     |
| HPB-ALL        | 83 ± 7  | 94 ± 4  | NT      |
| MOLT-4         | 0       | 93 ± 7  | NT      |
| HUT-78         | 0       | 81 ± 5  | NT      |
| CCRF/CEM       | 0       | 2 ± 2   | 102 ± 5 |

Bone marrow cells depleted of adherent cells were cultured in Petri dishes in medium containing 10-fold dilutions of crude SPN. Late CFU-GM-derived colonies were scored on day 7.

* Values represent the percentage of colony growth (mean ± SEM) in the experimental cultures as compared to control cultures (no SPN added). The number of colonies in control cultures was 254 ± 4 and 132 ± 7 per 10^6 mononuclear cells in two experiments with bone marrow samples from two different donors.

NT, not tested.

TABLE IX
Inhibition of Growth of Peripheral Blood CFU-GM after Brief (1 and 12 h) Exposure to T Leukemia-derived SPN

| SPN            | Dilutions | Numbers of colonies after exposure (h) to SPN: |
|----------------|-----------|----------------------------------------------|
|                |           | 1                        | 12                        |
| Control medium | —         | 397 ± 1*                 | 491 ± 9                   |
| Jurkat         | 10^{-1}   | 92 ± 20                  | 7 ± 7                     |
|                | 10^{-2}   | 396 ± 40                 | 6 ± 2                     |
| CCRF/CEM       | 10^{-1}   | 400 ± 20                 | 6 ± 4                     |
|                | 10^{-2}   | 416 ± 40                 | 213 ± 7                   |
| HUT-78         | 10^{-1}   | 271 ± 125                | 167 ± 35                  |
|                | 10^{-2}   | 412 ± 34                 | NT*                       |

Peripheral blood mononuclear cells depleted of adherent and phagocytic cells and of B and T lymphocytes were incubated for 1 or 12 h with 10^{-1} and 10^{-2} dilutions of crude SPN, washed extensively and plated at 150,000/Petri dishes. Colonies were counted after 14 d incubation.

* Values represent the number of CFU-GM-derived colonies scored in duplicate cultures (mean ± SEM).

NT, not tested.

studies showed that almost a total inhibition of growth was obtained with these SPN up to 10^{-3} dilution (Table X).

Partial Purification and Physicochemical Characterization. Partial characterization of the inhibitory activity was achieved by gel filtration on a Sephacryl S-300 column of ammonium sulfate-precipitated SPN (Fig. 6A). The SPN derived from Jurkat and CCRF/CEM cells were chosen because of their narrow and broad spectrum of reactivity, respectively, against the target cell lines studied (Table I). All fractions from each SPN were tested against the highly susceptible
TABLE X
Inhibition of Growth of Peripheral Blood BFU-E by
T Leukemia-derived SPN

| SPN          | Dilutions | Number of BFU-E-derived colonies |
|--------------|-----------|---------------------------------|
| Control medium | —         | 206 ± 5.3*                      |
| Jurkat       | $10^{-2}$ | 0                               |
|              | $10^{-3}$ | 0                               |
|              | $10^{-4}$ | 1 ± 1                           |
|              | $10^{-5}$ | 7 ± 4                           |
| CCRF/CEM     | $10^{-2}$ | 0                               |
|              | $10^{-3}$ | 0                               |
|              | $10^{-4}$ | 1 ± 1                           |
|              | $10^{-5}$ | 11 ± 2                          |

Peripheral blood mononuclear cells depleted of monocytes, T, and B lymphocytes were plated at $10^5$ cells/Petri dish in methylcellulose in the presence of various SPN concentrations, BPA, and erythropoietin. BFU-E-derived colonies were scored after 14 d of incubation.

*Values represent the number of colonies scored in duplicate cultures (mean ± SEM).

Daudi cells in a 5-d proliferation assay. The inhibitory activity produced by CCRF/CEM cells (TLSL$_{CEM}$) eluted in two peaks, one of $M_r$ 88,000, corresponding to fractions 18–21 (pool A), and one in the void volume of $M_r$ ≥ 300,000, which corresponded to fractions 9 and 10 (pool B) (Fig. 6A). In contrast, the activity produced by Jurkat cells (TLSL$_{JU}$) eluted in a single peak of $M_r$ 88,000, with no detectable activity in the void volume (Fig. 6A). When tested against the entire panel of hemopoietic tumor cell lines, neither the crude SPN nor the A or B pools derived from Raji cells inhibited proliferation (Table XI). Pool A from TLSL$_{JU}$ inhibited only the B lymphoblastoid lines Raji, Daudi, and BL2, which are sensitive to the crude Jurkat SPN (Tables I and XI). In contrast, both pools A and B of TLSL$_{CEM}$ inhibited the proliferation of all hemopoietic tumor cell lines. Whereas pool A of TLSL$_{CEM}$ remained active at a 1:100 dilution, neither pool B of TLSL$_{CEM}$ nor pool A of TLSL$_{JU}$ did (data not shown). Consequently, CCRF/CEM cells not only produced two forms of TLSL activity of high and low $M_r$, but generated more of the low-$M_r$ factor than Jurkat cells; furthermore, CCRF/CEM cells produced more low- than high-$M_r$ factor when grown in synthetic medium.

To determine whether the high-$M_r$ form of TLSL$_{CEM}$ is distinct from the low-$M_r$ form, or represents an aggregate, we generated TLSL$_{CEM}$-containing SPN in medium containing 5% FBS to favor aggregate formation before ammonium sulfate precipitation. An aliquot of resuspended precipitate was dialyzed and fractionated in PBS. Unlike the gel filtration of TLSL$_{CEM}$ generated in the absence of serum, substantially more activity remained in the high-$M_r$ fractions as compared to the low-$M_r$ fractions at 1:100 dilution (Fig. 6B). Furthermore, once formed, the aggregates could be disassembled by either detergent (Fig. 6B and C) or high salt (C), with the result that more TLSL activity appeared in the
Figure 6. (A) Inhibition of Daudi cell proliferation by fractionated TLSL from CCRF/CEM (■) and Jurkat (○) generated in synthetic medium. Crude SPN from Jurkat and CCRF/CEM cells grown in synthetic medium were precipitated in 85% ammonium sulfate, dialyzed, and placed on a 1.5 × 50 cm Sephacryl S-300 column equilibrated with PBS. 3-ml fractions were collected at a flow rate of 10 ml/h. Daudi cells (2 × 10⁴ cells/well in triplicate culture) were incubated in complete RPMI-1640 medium containing 1:10 dilution of each fraction, pulsed on day 5 with [³H]TdR and harvested 7 h later. Arrows indicate fractions containing blue dextran (BD) and molecular weight markers. (B) Inhibition of Daudi cell proliferation by fractionated TLSL_cE_M generated in serum-containing medium. Conditioned medium from CCRF/CEM cells containing 5% FBS was precipitated in 85% ammonium sulfate, dialyzed against the appropriate solution, and placed on a 1.5 × 50 cm Sephacryl S-300 column equilibrated with PBS (■) or PBS plus 0.02% Tween-20 (○). 3-ml fractions were collected at a flow rate of 10 ml/h. Every other fraction was tested for TLSL activity by incubating Daudi cells (2 × 10⁴ cells/well in triplicate culture) in complete RPMI-1640 medium containing 1:100 dilution of the fraction sample, followed by incubation and pulsing as above. (C) Inhibition of PHA-induced T lymphocyte proliferation by fractionated TLSL_cE_M generated in serum-containing medium. Conditioned medium of CCRF/CEM containing 5% FBS was precipitated in 85% ammonium sulfate, dialyzed against the appropriate solution, and placed on a 1.5 × 50 cm Sephacryl S-300 column equilibrated with PBS (■), PBS plus 0.5% NaCl (△), or PBS plus 0.02% Tween-20 (○). 3-ml fractions were collected at a flow rate of 10 ml/h, and every other fraction was tested for TLSL activity at 1:10 dilution, as above.
Table XI
Antiproliferative Activity of Fractions Obtained from Gel Filtration of
T Leukemia–derived SPN

| SPN       | Raji Crude | Daudi | BL2 | HL60 | ML3 | U937 | K562 | BV-173 |
|-----------|------------|-------|-----|------|-----|------|------|--------|
| Raji Crude| ≤1*        | ≤1    | ≤1  | ≤1   | ≤1  | ≤1   | ≤1   | ≤1     |
| Pool A    | ≤1         | ≤1    | ≤1  | ≤1   | ≤1  | ≤1   | ≤1   | ≤1     |
| Pool B    | ≤1         | ≤1    | ≤1  | ≤1   | ≤1  | ≤1   | ≤1   | ≤1     |
| Jurkat Crude | 83    | 93    | 40  | ≤1   | ≤1  | ≤1   | ≤1   | ≤1     |
| Pool A    | 99         | 97    | 97  | ≤1   | ≤1  | ≤1   | ≤1   | ≤1     |
| Pool B    | 100        | ≤1    | ≤1  | ≤1   | ≤1  | ≤1   | ≤1   | ≤1     |
| CCRF/CEM Crude | 94    | 97    | 94  | 81   | 99  | 89   | 99   | 97     |
| Pool A    | 99         | 99    | 98  | 42   | 57  | 51   | 95   | 88     |
| Pool B    | 74         | 66    | 50  | 55   | 41  | 13   | 55   | 82     |

* Target cells (2–4 × 10^3 cells/well) were incubated in microplates in the presence of 1:10 dilution of either crude SPN or their pooled A and B fractions (triplicate cultures). After a 5-d incubation at 37°C, [3H]-thymidine incorporation was measured.
* Percent inhibition of proliferation (mean from two or three experiments).
* Pool A (fractions 18–21) correspond to the peak of Mr 88,000 Pool B (fractions 9 and 10) correspond to the peak in the void volume of Mr >500,000.

fractions containing the smaller form of TLSL. These results suggest that the high-Mr component of TLSL-CEM is an aggregate of the low-Mr form and the relative amounts of each detected is dependent on the producer cell line, as well as the conditions used in production and processing of the supernatants.

Under standard column chromatography conditions, partially purified TLSL-ju did not bind to lectins covalently bound to Sepharose, including wheat germ, lens culinaris, and concanavalin A (data not shown).

Physicochemical studies were performed to analyze the stability of TLSL at various temperature and pH conditions. Titration experiments against Raji cells demonstrated that the activity of crude TLSL-CEM was (a) totally destroyed by exposure to 56°C for 30 min, or 75°C for 15 min, and (b) at least two to four orders of magnitude less active upon brief (10 min) exposure to low (3), and high (8.5) pH. Thus, TLSL is very sensitive to heat denaturation and unstable to extremes of pH. The isoelectric point of crude TLSL-CEM and TLSL-ju was found to be 5.2–5.3, as determined by flat-bed electrofocusing in Bio-lyte electrofocusing gel (Bio-Rad) with an LKB 2117 multiphor apparatus (LKB, Bromma, Sweden) (42).

Discussion

In this study we have shown that unstimulated human leukemia cell lines, (Jurkat, CCRF/HSB2, JM, HPB-ALL, MOLT-4, HUT-78, and CCRF/CEM) release into their medium a factor that inhibits hemopoietic cells of lymphoid, granulocytic, erythrocytic, and monocytic origin. The ability to produce this lymphokine is restricted to leukemic cells of the T lymphocytic lineage as no such factor could be detected in the conditioned medium of either monocytic, myeloid, or B leukemia/lymphoma cell lines. No correlation was found between the T cell phenotype of the lymphokine producer cell lines and their ability to
produce low or high titers of TLSL (data not shown). Titration studies against very sensitive target cells, such as Raji and PHA-activated lymphocytes, showed that the supernatant from the Jurkat cell line was the least active, and the ones from HUT-78, CCRF/CEM, and MOLT-4 cells were the most active. The lower activity of the factor produced by Jurkat cells can explain the apparent selective inhibition of proliferation of the most sensitive targets (B cell lines) and not of more resistant targets (cell lines of myeloid, erythroid, and monocytic lineage).

Studies reported so far (9-23) on the effect of inhibitory lymphokines on normal and leukemic hemopoietic cells have been incomplete, most of the studies limiting the target sensitivity analysis to B or T cell lines. TLSL is distinct from BIF because BIF is ineffective against MOLT-4, HSB2, Daudi, and K562 cells and is stable at pH 2 and at 56°C for 30 min (17). TLSL is biochemically separable from SIF (18-29 kD by gel filtration) and SIRS (110-150 kD by gel filtration) (12, 13, 23).

TLSL is not SISS-T (30-45 kD), which specifically suppresses T cell proliferation and requires adherent macrophage cells for its activity (15). TLSL is also distinct from SISS-B (60-80 kD) because the latter is stable at pH 2.5 and acts only on B cells arresting their Ig production (16). It is not LT because (a) LT is heat stable at 75°C over 15-min incubation, and is a glycoprotein (7, 8) while TLSL is not; (b) LT is directly cytotoxic whereas TLSL arrests cell proliferation without lysing the target cells; (c) mouse L cells, which are very sensitive to LT (6), are completely insensitive to TLSL; (d) monomeric LT has a Mr of 25,000 (8) rather than 45,000 as seen with TLSL.

TLSL has some characteristics in common with IDS, a factor released by activated T lymphocytes, such as the ability to inhibit [3H]TdR incorporation without causing cell death (10). IDS, however, is a glycoprotein, has a lower isoelectric point of 2.5, and its subunits are of 20 kD (43). It is impossible to compare the respective cell target range since most studies on IDS have been limited to HeLa cells and to mitogen-stimulated lymphocytes (10, 11). The designation of IDS has also been ascribed to the product of the macrophage-like cell line U937 (44). This monokine, released either spontaneously or in larger quantities upon mitogenic stimulation by U937 cells, shares with TLSL the characteristics of heat and acid lability, and the nontoxic mechanism of action; however, unlike TLSL, IDS has an Mr of 65,000 by gel filtration. Most important, its inhibitory activity is reversible, and is directed only against cells of lymphoid origin (mitogen-induced PBL and some neoplastic T and B cell lines).

TLSL is similar to CIL (25), based on its Mr, by gel filtration and on its ability to inhibit hemopoietic colony growth in a variety of leukemic cell lines. However, at difference with CIL, which in preliminary studies appeared to preferentially inhibit the proliferation of cells bearing surface class II HLA (Ia) antigens (25), TLSL inhibits cell growth regardless of the expression of HLA-D gene products on the target cells: the cell lines K562, HL60, ML3, U937, which do not express or only weakly express Ia antigens (45), were actually inhibited by most of the SPN in our study. The possibility that TLSL induces the expression of Ia antigens...
on myelomonocytic cells similar to that described for differentiation-inducing
factor (DIF) (45) and for IFN-γ (46, 47) can be ruled out, as immunofluorescence
analysis did not reveal the increase nor the appearance of any HLA-D gene
product on cells incubated with TLSL for up to 5 d (data not shown).

Finally, TLSL has some similarity with SAF, a factor produced by a 6TG-
resistant clone of the CEM cell line. TLSL, like SAF, inhibits [3H]TdR incorpo-
rati on by T cells at very low dilutions (up to 10⁻⁷) (18, 19), and arrests cell
proliferation in the G₁ phase of the cell cycle. The larger Mₙ (≥200,000) observed
for SAF (19) could be ascribed to aggregation phenomena of this lymphokine
when run on column chromatography in the presence of serum, as we observed
with TLSL. TLSL has the same isoelectric point as SAF and the same sensitivity
to heat inactivation. TLSL, however, clearly acts directly on the tumor cell lines,
while no information is available on the activity of SAF against these targets. In
addition, SAF acts by activating a suppressor factor produced by normal T cells,
whereas the mechanism by which TLSL inhibits the proliferation of mitogen-
or alloantigen-stimulated lymphocytes and of hemopoietic progenitor cells has yet
to be determined.

Although TLSL is produced in large amounts by T leukemia cell lines, some
of these producer lines are sensitive to high concentrations of the factor. The
intermediate producer CCRF/HSB₂ cell line was partly inhibited by the condi-
tioned medium of the high-producer HUT-78, CCRF/CEM, and MOLT-4 cell
lines, and the low-producer Jurkat was inhibited by all of the SPN except by its
own. Moreover, partially purified concentrated TLSL₀ inhibits all T cell lines
tested, including Jurkat cells themselves. This observation suggests that the T
leukemia cell lines producing TLSL might also produce adequate levels of
another factor that protects them from the inhibitory activity of TLSL.

The kinetics data indicated that, unlike human LT and tumor necrosis factor
(TNF), which are directly cytotoxic against neoplastic cells (8, 48), TLSL is
cytostatic, and cell death occurs subsequent to proliferative arrest. In fact, during
the first 2 or 3 d of incubation in the presence of TLSL, the cells grow at the
same rate as the control cultures; maximal inhibition of proliferation occurs on
day 4 or 5, and is not accompanied by a great loss of cell viability; the cells die
shortly after they have ceased dividing. A similar pattern of cell growth inhibition
was observed in methylcellulose under conditions of single-cell culture, indicating
that TLSL also abrogates the clonogenic potential of tumor cells.

With the exception of Raji cells that accumulated in the S phase, the tumor
target cells incubated with TLSL were arrested in the postmitotic, pre-DNA
synthesis interval corresponding to the G₀-G₁ phase of the cell cycle, in which
cellular commitment to proliferation is made (49). Neoplastic cells exposed to
IFN-γ and LT in combination also accumulate in that phase and cease dividing
(50); likewise, all terminally differentiated cells are arrested in the G₀ phase.
Maximal inhibition of proliferation occurred when TLSL was present through-
out the 5-d incubation; however, the proliferative ability of all the tumor target
cells was sharply reduced even after a very brief exposure to the inhibitory
lymphokine. This observation, together with the kinetics data of inhibition of
growth in liquid and semisolid cultures, indicated that, although the cells can
normally replicate for a few days after treatment, TLSL does bind readily to the
Cell membrane, and delivers the signal to cease dividing. The observation that overnight exposure of PBL to the T leukemia-derived SPN results in partial or total inhibition of their proliferative response to PHA might reflect TLSL-induced structural alterations on the cell surface that in some way abrogate lectin binding or \(^{3}H\)TdR uptake by lymphocytes. A similar mechanism could be responsible for the inability of blood and bone marrow CFU-GM or BFU-E to respond to CSF or BPA after overnight incubation with the T cell-derived SPN.

TLSL has now been purified to homogeneity and subjected to amino-terminal sequence analysis. Based on that analysis, on Mr determinations by SDS-PAGE and gel filtration, and on the functional and physicochemical characteristics presented in this study, TLSL can be distinguished from the three best-characterized human antitumor agents: LT (7, 8), IFN-γ (1, 2), and tumor necrosis factor (TNF) (51).

Summary

Human T leukemia cell lines spontaneously release into their medium a suppressor lymphokine, T leukemia-derived suppressor lymphokine (TLSL), able to inhibit proliferation, DNA synthesis, and colony formation in a variety of malignant hemopoietic cell lines, as well as in normal myelomonocytic progenitor cells from bone marrow and peripheral blood. Titration curves indicated that the inhibitory activity in the crude supernatant preparations ranged from \(10^{-8}\) to \(10^{-9}\); the supernatants from CCRF/CEM, HUT-78, and MOLT-4 cell lines were the most active, those from HPB-ALL, JM, and CCRF/HSBz displayed an intermediate activity, and the Jurkat supernatant was the least active. Target cell lines of B cell origin (Burkitt lymphomas) were more sensitive than granulocytic, monocytic, erythroid, and T cell lines. Partial purification by ammonium sulfate precipitation and column chromatography demonstrated that TLSL is a protein with an Mr of 88,000, as determined by gel filtration. A high Mr form (\(>300,000\)) was produced in serum-free medium by one of the most active producer cell lines (CCRF/CEM), and appeared to be an aggregate of the 88,000 Mr form. Neither the partially purified fractions obtained nor the crude supernatant preparations displayed antiviral activity or contained interleukin 2. Unlike lymphotoxin and tumor necrosis factor, TLSL is cytostatic: maximal inhibition of proliferation was observed 4–5 d after addition of crude supernatant to the target cells, and was not accompanied by a significant loss in cell viability. The antiproliferative capacity of TLSL was manifested both in suspension and methylcellulose cultures. Treated target cells accumulated either in the G1 or in the S phase of the cell cycle. The effect of TLSL on the target cells is irreversible: even brief (1 h) incubation of sensitive cells with TLSL resulted in inhibition of proliferation measured 5 d later. Although TLSL is produced by leukemic T cell lines, this lymphokine inhibits proliferation of normal peripheral blood T cells in response to mitogens or alloantigens: T lymphocyte activation was inhibited by all of the T cell supernatants tested. In contrast, when T cell lines were used as targets, no inhibition of proliferation was detected with two exceptions: the low producer Jurkat cell line was sensitive to all the T cell-derived supernatants, and the intermediate producer
CCRF/HSB₂ cell line was sensitive only to the three most active supernatants, CCRF/CEM, MOLT-4, and HUT-78.

The possible significance of TLSL and its relationship with other suppressor lymphokines previously described in other systems is discussed.

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