Shiga-like toxin-1 (SLT-1) is a bacterial toxin that kills cells by inhibiting protein synthesis. SLT-1 is composed of one cytotoxic A-subunit and five B-subunits that bind to CD77, a cell-surface glycolipid. In the human hematopoietic system, CD77 expression is restricted to a subset of activated B cells and derived cancers. Here we report that SLT-1 treatment of murine bone marrow ex vivo effectively cures severe combined immunodeficient mice of a human B-cell lymphoma xenograft while sparing normal hematopoietic precursor cells. Flow cytometry results using fluorescein isothiocyanate-labeled SLT-1 B-subunit show the high prevalence of expression of SLT-1 receptors (CD77) in human non-Hodgkin’s lymphomas, especially follicular lymphomas. These results suggest the use of SLT-1 for the purging of human bone marrow before autologous bone marrow transplant in the case of CD77+ B-cell lymphomas as just one of many possible uses.© 1996 by The American Society of Hematology.
Cambridge, MA), and 50 U of human IL-3. Plates were incubated in a humid atmosphere at 37°C containing 5% CO₂ for the indicated periods of time. Cytocenies of greater than 50 cells were counted under a microscope.

**Immune reconstitution experiments.** SCID mice (C.B-17 scid/ scid) were bred and maintained in our own pathogen-free defined flora colony. For transplant experiments, only female mice at 8 to 13 weeks of age were used. Female BALB/c ByJ mice 6 to 8 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME) as a 'congenic' strain to SCID mice used as donors for the BMTs. All animal experiments were performed according to the guidelines of the Medical Research Council of Canada. The holotoxin SLT-1 (M, 76,661) was purified from *Escherichia coli* culture transformed with the SLT-1-coding plasmid.17 BM was obtained from BALB/c ByJ mice and treated with or without 1 µg/mL SLT-1 for 1 hour at 37°C and washed. BM cells (2 × 10⁶) were injected into irradiated SCID mice. Ten weeks posttransplant, peripheral blood (PB) was obtained from the tail vein and analyzed for the presence of T and B cells. Reconstitution of SCID mice with BM from BALB/c ByJ mice was verified by flow cytometry analysis of 50 µL of PB from the reconstituted SCID mice, with untreated SCID mice and BALB/c ByJ mice as controls. The appearance of CD3⁺ cells (mature T cells) in the periphery was detected with a fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse T3 complex CD3ε monoclonal antibody (MoAb) (Cedarlane; Hornby, Ontario, Canada) and phycoerythrin-conjugated anti-B220 MoAb (PharMingen, San Diego, CA). Flow cytometry was performed on a Becton Dickinson FACSscan with Lysis II software (San Jose, CA). Data from 10,000 events (mononuclear cells) was collected.

**SLT-1 purging experiments.** The human Burkitt’s lymphoma cell line, Daudi, was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and was maintained in α-MEM medium with 20% heat-inactivated FCS (CELLect GOLD; ICN Flow, Mississauga, Ontario, Canada). BM was isolated under aseptic conditions from the femora and tibiae of untreated SCID mice by flushing with a 25-gauge needle and IMDM media containing 5% FCS. BM was mixed 2:1 with or without Daudi cells, and treated with or without 10 ng/mL SLT-1 for 60 minutes in culture dishes at 37°C. Cells were washed twice in Hank’s balanced salt solution without CaCl₂ and MgCl₂ (HBSS−) supplemented with 1% FCS and resuspended in HBSS−/FCS solution so that each mouse received 2 × 10⁶ nucleated BM cells with or without 1 × 10⁶ viable (dye-excluding) Daudi cells in 200 to 300 µL. Cells were mixed and split into equal volumes for the various experimental treatments so that the groups received an equal number of cells. SCID mice received a sublethal dose of γ-irradiation (0.4 Gy) from a 137Cs source (dose rate = 0.54 Gy/min) just before injection of the BM.99 Mice were monitored daily for signs of disease. Animals were killed at signs of paralysis and the time recorded.

**Screening of human cancers for SLT-1 receptors by flow cytometry.** The B-subunit of SLT-1 was purified from an *E. coli* culture transformed with the B-subunit-coding plasmid, pJLB122, as previously described.29 FITC (Molecular Probes, Eugene, OR) was added directly to purified SLT-B dissolved in phosphate-buffered saline (PBS), pH 7.4. Free FITC was removed by chromatography on a Sephadex G-50 (Pharmacia, Baie d’Urfé, Canada) column equilibrated in 50 mmol/L NH₄HCO₃. The orange-colored peak eluting in the void volume of the column was collected, lyophilized, and stored at –5°C. The FITC-SLT-B conjugate was resuspended to a concentration of 0.25 mg/mL in PBS or water. Samples were stained with a 1:50 to 1:75 dilution of FITC-SLT-B and analyzed by flow cytometry using a Becton Dickinson FACSscan flow cytometer. The diagnosis was based on several criteria including histology, cytogenetics and was made by the pathologist in the group (B.P.).

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**RESULTS**

**SLT-1 cytotoxicity.** SLT-1 binds to a glycolipid present on colonic and kidney endothelial cells, called globotriosylceramide (Gb₃), which permits its internalization and leads to cell killing. This glycolipid is referred to as the CD77 antigen in the hematopoietic system and shows a restricted pattern of expression limited to a subset of activated B cells in the germinal (follicular) center.9,10 CD77 expression is prevalent in certain hematologic cancers of B cells11,14 such as Burkitt’s lymphoma, represented here by the cell line, Daudi. The sensitivity of Daudi cells toward the toxin was tested using purified SLT-1. The 50% inhibitory dose (ID₅₀) for the toxin was found to be 0.3 ng/L (4 × 10⁻¹³ mol/L) as measured by protein synthesis inhibition that precedes cell death in this case (Fig 1). To verify that the murine BM cells demonstrated minimal toxicity toward SLT-1, BM cells were cultured in an in vitro colony-forming assay in the presence or absence of toxin. The results presented in Table 1 show that the toxin was not toxic to the most primitive murine BM precursor cells seen in this assay. A similar experiment with human BM from a single acute myelogenous leukemia patient also showed little or no toxicity at the

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**Fig 1.** Dose-dependent inhibition of protein synthesis in Daudi cells by Shiga-like toxin 1 (SLT-1). Uptake of (¹⁴C)-leucine by Daudi cells was used as a measure of protein synthesis. Daudi cells (50,000 cells/well) were incubated for 24 hours with increasing concentrations of SLT-1, ranging from 10⁻³ g/L to 10⁻¹⁶ g/L. The incorporation of tritiated leucine over a period of 24 hours was measured by harvesting cells onto filters at the end of the incubation time and counting the recovered radioactivity. Counts associated with cells incubated with tritiated leucine in the absence of toxin were taken to represent maximal protein synthesis activity (100%) which corresponded to an absolute value of 10,200 cpm. Background counts (no cells) were only 1% of the maximal uptake (cells with no toxin).
highest dose tested (1 μg/mL) for day 7 colony-forming unit (CFU) or day 16 burst-forming unit-erythroid (BFU-E) and day 16 CFU in culture (CFU-C).

**SLT-1 effect on immune reconstitution.** SLT-1-treated or untreated BM cells were transplanted into irradiated SCID mice to verify their reconstitution in an in vivo setting. SCID mice lack circulating mature B and T cells. BM from an immunocompetent ‘congenic’ strain of mouse (BALB/c ByJ) was treated or not with SLT-1 in vitro and used to reconstitute SCID mice. The appearance of mature B and T cells, indicative of reconstitution by BALB/c ByJ BM precursors, was monitored by flow cytometry using antibodies to CD3 (T cells) and B220/CD45R (B cells). SCID mice transplanted with the BALB/c ByJ BM had a reconstituted immune system at 10 weeks posttransplant (Fig 2A) since their CD3 profiles (68%) were identical to those of a BALB/c ByJ mouse (57%). No obvious differences could be observed in the percentages of T cells in the reconstituted mice that had received marrow after SLT-1 treatment (61%) or no treatment (68%). Evidence of reconstitution of the B-cell lineage was similarly confirmed by flow cytometry (B220/CD45R; Fig 2B).

**SLT-1 purging of human lymphomas ex vivo.** Purging experiments were then initiated in SCID mice which served as a transplant host for the human xenograft. This model has a well-defined endpoint, ie, hind-leg paralysis of SCID mice caused by the dissemination and invasion of the spinal cord by the lymphoma.14,15 BM was obtained from SCID mice, seeded or not with Daudi cells (33% of total cells which represents a high tumor burden), purged with or without 10 ng/mL of SLT-1 for 60 minutes at 37°C, washed, and injected into irradiated SCID mice. Mice were examined daily for signs of disease and the period of disease-free survival (paralysis-free) noted. The Kaplan-Meier plot (Fig 3) illustrates the rapid onset of cancer symptoms (paralysis at days 38 through 49) for our longest-running experiment for the group of mice injected with BM and 1 million untreated Daudi cells (disease control). The purging of Daudi-contaminated BM with SLT-1 has lead to a large increase in disease-free survival (and cure), as this group is still alive and disease-free 9 months past the disease control group median period for disease-free survival.

**Screening of human cancers for SLT-1 receptors.** The B-subunit of SLT-1 (SLT-B; binding subunit) represents the component of SLT-1 that binds to CD77. It was labeled with FITC and used to screen patient samples (Department of Oncologic Pathology, Princess Margaret Hospital, Toronto). One hundred ten patients were examined. The percentage of CD77+ cells for a gated population of cells (eg, CD19+ cells or lymphocytes for lymphomas, or blasts for leukemias) were plotted for the various cancers diagnosed by the pathologist (Fig 4). An average of 3% ± 4% positive cells was observed for the control group (n = 11) consisting of noncancerous patients. Cell preparations with 15% (3 SDs above the mean background) of their population staining positively for this marker were defined as ‘true’ positives. The most striking result was that 44% of malignant lymphomas (MLs) (23 of 52 patients) were positive. Sixty-nine percent of patient samples obtained from the follicular lymphoma subgroup of ML stained positively (11 of 16) with the FITC-SLT-B probe.

**DISCUSSION**

The goal of this study was to establish the potential of a natural toxin known as Shiga-like toxin 1 (SLT-1) in purging B-cell lymphomas from BM. The Burkitt’s lymphoma cell line, Daudi, is sensitive to SLT-1 (Fig 1, ID50, 0.3 pg/mL), as are the majority of Burkitt’s lymphoma cell lines, eg, Ramos, BL41, BL2, Raji, Mutu I, and Daudi.21 This effect is not unique to Burkitt’s lymphomas because a number of other human tumor cell lines and normal cells are killed by SLT-1. These cell lines include neoplastic and transformed B cells, such as the hairy cell leukemia cell line GASH (ID50, 0.3 ng/mL) and the lymphoblastoid cell lines HSC-3 (ID50, 1.5 ng/mL) and C11 (ID50, 2.5 ng/mL). v an ID50 of 0.01 ng/mL for Daudi in this study).22 Lipopolysaccharide-stimulated tonsillar B cells and normal germinal center CD77+ B cells (ID50 < 10 ng/mL)22,23 are also sensitive to the action of the toxin. Some other CD77+ nonlymphoid cell lines show sensitivity to SLT-1, including HeLa cells (ID50, 10 to 45 pg/mL) and Vero cells (African green monkey kidney cell line; ID50 of 1.0 pg/mL24), which have been traditionally used in in vitro assays to monitor the cytotoxic activity of Shiga and SLT-1 toxins. Other cell lines include the human monocytic leukemia cell line THP-1 (ID50, 18 pg/mL)24 and CD77+ germ cell tumors such as the human ovarian cell line SKOV3 (ID50, 100 ng/mL).25

An important criteria for using SLT-1 in BM purging experiments is that BM progenitors show no or little sensitivity to the purging agent even at high doses. Treatment of
murine BM with even 10 µg/mL of SLT-1 (>10⁷ times the ID₉₀ dose for Daudi cells, Fig 1) resulted in only a 40% reduction in the number of colonies (Table 1). This result was expected because the expression of CD77 has not been detected in human BM by immunochemistry, nor has SLT-1 shown any alarming toxicity toward human BM precursor cells (ID₉₀ > 1 µg/mL) in vitro. SLT-1 toxicity against BM precursor cells was also tested in a setting (immune reconstitution) that more closely resembles the ABMT procedure. Flow cytometric analyses (Fig 2) illustrate that PB from control SCID mice had virtually no CD3⁺ or B220⁺ cells whereas a control BALB/c ByJ had a large percentage of CD3⁺ cells (57%) and B220⁺ cells (40%) for the lymphocyte gated-population. The lymphocyte population in BALB/c ByJ or reconstituted SCID mice was 58% to 65% of the total mononuclear cell population in comparison to only 16% to 17% for age-matched control SCID mice (data not shown). No differences were seen between mice receiving SLT-1-treated marrow or untreated marrow (Fig 2). The flow cytometric data agree with published values of differential counts of leukocytes in the PB of SCID mice and immunocompetent C.B-17 mice and show the safety of using SLT-1 as a purgative in the mouse and, most likely, in humans. The appearance of B220⁺ cells, B cells, in the peripheral circulation along with CD3⁺ T cells (which indicates that Ig gene rearrangement has taken place) suggest that these cells are...
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The Burkitt's lymphoma cell line, Daudi, was chosen as a model for NHL. The appearance of cancer symptoms (Fig 3) in our mice agrees with expected results for the injection of 10^6 Daudi cells. The implantation of only 100 Daudi cells has been shown to give rise to hind-leg paralysis by about day 90 (mean survival time). The injection of 10^6 SLT-1-intoxicated Daudi cells has resulted in a tripling of the mice disease-free survival period, which suggests that at least 4 logarithmic units of Daudi cells have been purged from the BM. The experiment has been repeated twice more with similar results.

Previous work by other groups identified the tissue and cell distribution of CD77 on normal and neoplastic tissues using antibodies. Because the structural determinants of CD77 recognized by MoAbs are likely different than those recognized by the toxin itself, we probed with the toxin B-subunit for the presence of SLT-1 receptors on human cells derived from cancer patients that might benefit from ABMT. These results emphasize the prevalence of this marker for MLs, especially follicular lymphomas, and its virtual absence from other hematologic (myeloid) cancers and normal samples. It agrees with previous results with anti-CD77 antibodies, which show a high frequency (~60%) of expression for NHL.

The two main approaches to BM purging are chemotherapeutic regimens and immunologic therapies that involve MoAbs or cytokines. Other approaches include physical, biological, and other affinity-binding techniques to selectively eliminate tumor cells from the graft. With the negative selection approaches (removal of tumor cells or cell-killing strategies), it is feasible to eliminate up to 6 log units of tumor cells. This is especially true when combinations of either MoAbs and pharmacologic compounds are used, or when multiple rounds of immunomagnetic bead separations are performed. An alternate approach is to positively select for hematopoietic reconstituting cells (enrichment for CD34^+ cells), which results in a 2- to 3-log--fold depletion of tumor cells. The performance of such an enrichment strategy can potentially be further enhanced by a factor greater than 100 (2 log units) by using apheresis products (PB stem cells) over BM for some malignancies. Unlike immunotoxins, the native SLT-1 toxin has already been optimized for cellular internalization and trafficking as well as for the translocation of its enzymatic A-chain leading to the efficient intoxication of cells. In the case of immunotoxins, researchers have to determine empirically which antibodies will be efficiently internalized. The intracellular routing of immunotoxins (endosomal and lysosomal compartments for receptor-mediated endocytosis) is different than that of Shiga and plant toxins (optimized for retrograde transport to the luminal compartment of the endoplasmic reticulum), which will probably lead to lower efficiency of intoxication.

In comparison with chemotherapeutic drugs, SLT-1 offers many advantages. It can theoretically kill a cell at a low dose of 1 molecule per cell. However, a more likely number is 10 to 200 molecules per cell due to the efficiency of translocation. SLT-1 target cells are typically 1,000 times functional. We conclude from our reconstitution experiments that SLT-1 was not toxic to the common lymphoid precursor of B and T cells.

![Fig 3. Disease-free survival of SCID mice transplanted with SLT-1 purged marrow. Disease-free survival was plotted as the time to paralysis of SCID mice transplanted with Daudi cells (1 x 10^6) treated with or without 10 ng/mL SLT-1 (37°C, 60 minutes). Mice were injected via the tail vein with either BM cells (sterility control, ▽), BM cells seeded with Daudi cells (positive disease control, ◆), SLT-treated BM (washing control, ■), or with SLT-treated BM and Daudi mix (purged marrow/treatment group, ○) without toxin-neutralizing antibody. The CD77 receptor on Daudi cells (Daudi cells, ◇) was mixed with a toxin-neutralizing polyclonal antibody (100 µL of antiserum for 200 µL of cells) after treating the BM with the toxin before injection. One mouse out of 10 in the purged groups died on day 98 (△). This animal showed no signs of paresis or paralysis. Its death was attributed to natural causes, although the cancer cannot be ruled out as a cause of death.](image)

![Fig 4. Detection of CD77^+ cells in human hematologic cancers. Flow cytometry was performed with FITC-labeled SLT-1 B-subunit. The percentage of positive cells from the gated populations (CD19^+ or lymphocytes or blasts) for the various patient samples (●, PB, and biopsies such as fine-needle aspirates, lymph node, and BM biopsies) are shown. A cut-off of 15% positive cells is indicated to distinguish sample preparations with a high number of positive cells in relation to other patient samples. The mean percentage of stained cells for samples below and above the cut-off is also indicated (—). Abbreviations: Control, noncancerous controls; MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; ML, malignant lymphoma which includes NHL, acute lymphocytic leukemia and B-cell chronic lymphocytic leukemia. Samples from patients diagnosed with follicular lymphoma are indicated by square symbols (■) in the ML group.](image)
more sensitive than nontarget cells, which surpasses the differential sensitivity of chemotherapeutic agents for tumor cells versus normal ones.

SLT-I represents an ideal purging agent for the following reasons. It is cytotoxic throughout the cell cycle and differs in cell-cycle dependence patterns from that of conventional chemotherapeutic drugs. It possesses an impressive ability to eliminate clonogenic tumor cells (greater than the detection limit of the assays used here, i.e., >4 to 5 log units of cell killing). It shows no toxicity against normal BM progenitors, sparing stem cells. It is very soluble in most aqueous media and can be easily removed before reinfusion. Finally, SLT-I possesses a mechanism of action (which kills cells through the enzymatic inactivation of ribosomes) that is distinct from those of conventional therapeutic drugs, suggesting that known mechanisms of drug resistance will not counter the action of SLT-I. This feature may allow the eradication of drug-resistant clones that would otherwise lead to relapse. SLT-I may also potentiate the action of conventional drugs, as is the case for immunotoxins.

In conclusion, we have shown in an animal model that treatment of a lymphoma with a single biologic agent ex vivo can result in cure. We have demonstrated the high expression of SLT-1 receptors in MLs, especially follicular lymphomas. Follicular lymphomas are classified as indolent low-grade lymphomas for which there is no curative treatment. The promising results in our animal studies warrant further preclinical studies of human samples. SLT-1 treatment in the context of ABMT and BM purging may provide a cure for follicular lymphoma patients. This application represents only one of the many possible future uses and strategies for SLT-I in cancer therapy. For example, SLT-I may be used in combination with chemotherapeutic drugs or immunotoxins to further increase its efficacy, or as a purging agent for solid tumors, such as germ cell tumors which also express Gb3 (SLT-1 receptors). As the clinical use of PB stem cell transplants increases, there will be a need for purging tumor cells from the graft. If the ex vivo use of SLT-I proves safe and efficacious then it may potentially be used in vivo to eliminate residual disease after chemotherapy.

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