Immunomodulation by Various Nitrosoureas and Its Effect on the Survival of the Murine Host Bearing a Syngeneic Tumor

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ABSTRACT

Chemotherapeutic efficacies of the nitrosoureas 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), chlorozotocin (CLZ), and streptozotocin (STZ) were investigated against the LSA tumor which is syngeneic to C57BL/6 mice. It was observed that a single injection of 20 mg/kg body weight of BCNU or CLZ, even at an advanced stage of tumor growth, completely cured >90% of the tumor-bearing mice. Furthermore, BCNU-cured or CLZ-cured mice could specifically reject secondary rechallenge with the LSA tumor. In contrast, a single dose treatment with STZ at 20–200 mg/kg body weight failed to cure the tumor-bearing mice (0% survival). The failure of STZ to cure tumor-bearing mice was next addressed considering three possible mechanisms: (a) STZ was less tumoricidal; (b) STZ suppressed the immunity of the host; and (c) STZ failed to elicit tumor-tumor suppressor T-cells. The failure of STZ to cure tumor-bearing mice was not totally related to its tumoricidal properties since STZ at higher doses did possess significant tumoricidal activity in vitro and in vivo, comparable to that of BCNU or CLZ. When spleen cells from normal mice treated with BCNU, CLZ, or STZ were assayed for their responsiveness to the T-cell mitogens concanavalin A or phytohemagglutinin, it was observed that STZ was in fact less immunosuppressive than BCNU or CLZ. The fact that STZ did not suppress the immunity of the host was also suggested by the findings that BCNU-cured mice treated with STZ or CLZ could still reject secondary rechallenge with the specific tumor LSA. Following treatment of tumor-bearing mice with BCNU or CLZ, tumor-specific delayed type hypersensitivity responses were demonstrable in these mice but not in STZ-treated mice. The inability of STZ-treated tumor-bearing mice to elicit a delayed type hypersensitivity response was not due to selective depletion or delayed type hypersensitivity-inducing CD4+ T-cells but was probably due to failure of STZ to eliminate tumor-specific suppressor cells. Together these findings suggested that the failure of STZ to cure LSA tumor-bearing mice was not due to lack of tumoricidal activity or related to suppression of tumor-specific effector T-cell function but may be due to the failure of STZ to eliminate tumor-specific T suppressor cells. The present study suggests that the outcome of chemotherapy with nitrosoureas depends, in addition to the tumoricidal activity of the drug, on the immunomodulating action on the immune mechanisms of the host.

INTRODUCTION

NU3 have been used extensively as antineoplastic agents in the treatment of a variety of human cancers and experimental tumors (1–5). The toxic effects of NUs have been attributed to the alkylating properties of the carbonium ion and the carbamoylating properties of the isocyanate that are produced following degradation (6). The tumoricidal activity is believed to be due to the alkylating property of NUs whereas the severe, delayed, cumulative myelosuppression seen is due to their carbamoylating property (6). The carbamoylating activity of NUs has been found to be reduced following attachment of the NU to the C-2 position of glucose. STZ and CLZ are two nitrosourea analogues having low carbamoylating activity (6–8). However, they differ from each other in that STZ has a methylnitrosourea moiety attached to the C-2 position of glucose whereas CLZ has a chloroethyl nitrosourea group attached to the glucose (6–8). Due to low carbamoylating activity, CLZ and STZ are believed to be less toxic to the bone marrow when compared to BCNU, while retaining similar antimtumor activity (6–8).

The use of tumoricidal drugs to treat mice bearing syngeneic tumors has not, in general, ensured complete cure, because the escape of even a few tumor cells from the cytotoxic action of the drug would be sufficient to cause death. Thus, in addition to a drug-mediated reduction of tumor load it would be beneficial to have the host’s immune mechanisms operating simultaneously to ensure that no tumor cells survive. This means that the tumoricidal drug should not have a negative effect on the host’s effector T-cell functions.

In our earlier studies we observed that treatment of C57BL/6 mice bearing a syngenic tumor, LSA, with BCNU resulted in 90–100% survival of the mice and, more interestingly, 100% of the cured mice rejected secondary rechallenge with the homologous tumor but not with a heterologous syngeneic tumor such as EL-4 (9). Furthermore, it was observed that BCNU treatment was effective only in normal tumor-bearing mice but not in irradiated or nude tumor-bearing mice, thereby suggesting that an intact T-cell-mediated immune system was essential for effective treatment with BCNU (10). BCNU was also found to positively modulate the antitumor immune responses by depleting tumor-specific T-suppressor cells and enhancing the activity of macrophages and lymphokine-activated killer cells (11). These findings helped to explain why a single-dose treatment with BCNU was so highly effective even against advanced stages of tumor growth and why BCNU-cured mice successfully resisted subsequent rechallenge with the homologous tumor. In the present study we compared the antitumor activities and immunomodulating properties of CLZ and STZ with those of BCNU and correlated these findings with the survival of C57BL/6 mice challenged with the syngeneic LSA tumor. Our data suggested that for the nitrosoureas to be effective in mediating tumor rejection, in addition to their primary tumoricidal activity, their effect on the host’s immune system plays a crucial role.

MATERIALS AND METHODS

Animals. Adult female C57BL/6 mice were obtained from the National Cancer Institute, Bethesda, MD.

Tumors. LSA, a spontaneous thymic lymphoma syngeneic to C57BL/6 mice, and EL-4, a chemically induced leukemia also syngeneic to C57BL/6 mice, were maintained in ascites form by serial i.p. passages of 10⁶ tumor cells and in vitro by subculturing the tumor cells at 37°C at a concentration of 10⁵/ml in 75-cm² tissue culture flasks (Costar, Cambridge, MA) in culture medium consisting of RPMI 1640 (Gibco Laboratories, Grand Island, NY), 10% fetal calf serum (Gibco Laboratories, Grand Island, NY), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 40 μg/ml gentamicin sulfate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution, and 10% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY).

Drugs. BCNU, CLZ, and STZ were obtained from the National...
Cancer Institute and stored at −20°C. They were weighed immediately before use.

**In Vivo Drug Treatment.** BCNU was dissolved in absolute ethanol and diluted further with PBS, pH 7.2, to obtain a final ethanol concentration of 0.09% (9–11). CLZ and STZ were dissolved in a small volume of citrate buffer, pH 4, and further diluted in PBS. BCNU was injected i.p. at a concentration of 20 mg/kg body weight, 5 days after 1×10⁶ LSA tumor cell inoculation. CLZ and STZ were also given i.p. at varying doses 5 days or at different time intervals following tumor cell injection. Groups of 5–10 mice were used in all experiments. Control groups received only the vehicle.

**DTH Reaction.** The DTH reaction to LSA was studied as described at length elsewhere (10). Briefly, 1×10⁶ LSA tumor cells irradiated at 5000 rads and suspended in PBS were inoculated into the right hind footpads of mice. The left footpads received injections of PBS only. Twenty-four h later, the footpad swelling was measured using a dial gauge micrometer (Dyer Co., Lancaster, PA) and expressed in units (1 unit = mm × 10⁻²).

**Mitogenic Stimulation of Lymphocytes.** Spleen cells were removed aseptically from mice, single cell suspensions were made and erythrocytes were removed by lysis with 0.83% solution of ammonium chloride. Cell proliferation to mitogens was measured using a [³H]thymidine incorporation assay (12). Spleen cells were plated in triplicate at a concentration of 2×10⁶ cells/well in 96-well round bottomed plates in 200 μl of culture medium with the mitogens, Con A or PHA, at final concentrations of 2 or 10 μg/ml, respectively. Cultures were incubated at 37°C for 48 h. Cells were pulsed with 2 μCi of [³H]thymidine (New England Nuclear, Boston, MA) 18 h prior to cell harvesting. Cells were harvested onto glass fiber filter paper and counted with scintillation fluid in a liquid scintillation counter. (Beta trac 6895; TM Analytic, Inc., Elk Grove Village, IL). The results were expressed as mean cpm ± SEM of triplicate cultures.

**In Vitro Inhibition of Tumor Cell Proliferation.** LSA tumor cells (2×10⁶/well) were cultured in triplicate in 96-well plates in the presence of 0.01 to 20 mM concentrations of BCNU, CLZ, or STZ. As a control, the appropriately diluted vehicle was used. Forty-eight h later, the cells were harvested. [³H]thymidine was added to the cultures 18 h prior to harvesting.

**Fluorescent Staining for CD4+ T-Cells.** Flow cytometric analysis of CD4+ T-cells was performed as described at length elsewhere (13). Briefly, 1 million spleen cells from nitrosourea-treated or untreated tumor-bearing mice were incubated with PE-conjugated anti-mouse CD4 antibodies (Becton Dickinson, Mountain View, CA). The cells were suspended in 0.1 ml of PBS containing 0.02% sodium azide and 5 μl of PE-conjugated anti-CD4 antibodies. The cells were incubated on ice for 30 min and washed 3 times, and 10,000 cells were analyzed by flow cytometry (Epics V; Coulter Electronics, Hialeah, FL).

**Statistical Analysis.** Statistical significance of differences between groups was studied using Student’s t test and P < 0.05 was considered to be statistically significant.

**RESULTS**

**Effect of Treatment of Tumor-Bearing Mice with BCNU, CLZ, or STZ.** Groups of 6–10 C57BL/6 mice were given injections of 10⁶ live LSA tumor cells i.p. On day 5, all mice received 20 mg/kg of BCNU or CLZ. The control mice received the respective vehicle used to dissolve the drugs. The data shown in Fig. 1A demonstrated that untreated tumor-bearing mice died of the tumor by day 17. In contrast, 100% of the tumor-bearing mice treated with 20 mg/kg of CLZ survived for over 60 days. Twenty mg/kg of BCNU could also cure >90% of tumor-bearing mice, similar to our earlier observation (9–11). These data suggested that CLZ when used at a dose and time similar to that for BCNU was as effective as BCNU in curing a high percentage of tumor-bearing mice. Furthermore, tumor-bearing mice cured following treatment with CLZ, when rechallenged with 10⁶ LSA tumor cells i.p. 30 days later, could reject the homologous tumor challenge (Fig. 1A) but not a heterologous syngeneic tumor (EL-4) challenge (data not shown).

When LSA tumor-bearing mice were similarly treated by injection of 20 mg/kg of STZ on day 5, it was observed that all mice died by day 18, when compared to the control group of mice which died by day 13 (Fig. 1B). Presuming that STZ was not cytotoxic to the tumor cells at 20 mg/kg, increasing doses of STZ were administered to the tumor-bearing mice on day 5. It was observed that 100 or 200 mg/kg of STZ also failed to cure the tumor-bearing mice (Fig. 1B). It was interesting to note that higher doses of STZ such as 100 or 200 mg/kg although unable to cure the tumor-bearing mice, were able to increase their mean survival time from 12.5 ± 0.2 days (control) to 20.7 ± 0.6 and 23.7 ± 0.8 days, respectively (P < 0.05). It should be noted that injection of STZ at different days following tumor inoculation, such as day 1 or 10, had a similar effect since all these mice died of tumor growth (data not shown). Also, 200 mg/kg of STZ is the maximum tolerated dose of STZ by normal mice; therefore we could not increase the dose of STZ used any further.

**Tumoricidal Effect of BCNU, CLZ, or STZ on LSA Tumor Cells in Vivo and in Vitro.** To investigate whether the failure of STZ to cure LSA-bearing mice was related to its inability to kill tumor cells efficiently, comparative tumoricidal activity of BCNU, CLZ, and STZ was investigated in vivo and in vitro. LSA tumor-bearing mice were given injections of BCNU, CLZ, or different concentrations of STZ as described earlier. Twenty-four or 72 h later, these mice were given injections of 5 ml PBS, the peritoneal cavity was vigorously shaken, and the peritoneal fluid was aspirated out. The viable tumor cells were next enu-
merated by the trypan blue dye exclusion test. The data depicted as total viable cells/ml of the peritoneal fluid (Fig. 2) revealed that 20 mg/kg of BCNU or CLZ was highly tumoricidal and reduced the tumor load significantly ($P < 0.05$). Although 20 mg/kg of STZ could also reduce the tumor load significantly, it was less effective than BCNU or CLZ. At higher doses (200 mg/kg), however, the tumoricidal activity of STZ was comparable to that of BCNU or CLZ ($P > 0.05$). These data therefore suggested that STZ at 200 mg/kg body weight had in vivo tumoricidal activity comparable to that of 20 mg/kg body weight of BCNU or CLZ, although it should be noted that STZ at 200 mg/kg failed to cure LSA tumor-bearing mice.

Similar results were obtained when viable tumor cells were enumerated by trypan blue dye exclusion test. The data from 3 mice received only the vehicle. Twenty-four h later, these mice were given injections of 5 ml of PBS and the peritoneal fluid was aspirated out. The viable tumor cells were enumerated by trypan blue dye exclusion test. The data depicted in Fig. 2 revealed that 20 mg/kg of BCNU or CLZ was highly tumoricidal and reduced the tumor load significantly ($P < 0.05$). Although 20 mg/kg of STZ could also reduce the tumor load significantly, it was less effective than BCNU or CLZ. At higher doses (200 mg/kg), however, the tumoricidal activity of STZ was comparable to that of BCNU or CLZ ($P > 0.05$). These data therefore suggested that STZ at 200 mg/kg body weight of BCNU or CLZ, although it should be noted that STZ at 200 mg/kg failed to cure LSA tumor-bearing mice.

Effect of Treatment of Mice with BCNU, CLZ, or STZ on T-Cell Responses to Con A or PHA in Vitro. To investigate whether STZ had any immunosuppressive effect, normal mice were treated with 20, 100 or 200 mg/kg body weight of STZ and compared with 20 mg/kg of BCNU or CLZ treatment. Two days later, spleen cells were harvested and stimulated with the T-cell mitogens Con A or PHA. As demonstrated in Fig. 3, 20 mg/kg of BCNU or CLZ suppressed the T-cell responses to Con A and PHA significantly ($P < 0.05$). In contrast, 20 or 100 mg/kg of STZ failed to suppress the T-cell responses ($P > 0.05$) and 200 mg/kg of STZ inhibited the response only partially (Fig. 3). Collectively these data suggested that STZ was less immunosuppressive when compared to BCNU and CLZ.

Effect of STZ or CLZ on LSA Tumor-specific Immune Response in Vivo. Although BCNU, CLZ, and high doses of STZ suppressed the T-cell responsiveness to Con A or PHA in vitro, it was not clear how these data would reflect on the antitumor T-cell-mediated immunity in vivo. To investigate this possibility, we used mice with our earlier observation that BCNU-cured mice rejected secondary rechallenge with the LSA tumor cells and that this rejection was mediated by CD4+ and CD8+ T cells (10). We therefore treated BCNU-cured mice with 20 mg/kg body weight of CLZ or 200 mg/kg of STZ or with the vehicle (control) used for dissolving CLZ and STZ. Forty-eight h later, these BCNU-cured mice were given injections of $10^7$ LSA tumor cells. As a control, normal mice were given injections of $10^7$ LSA tumor cells. The data shown in Fig. 4 demonstrated that BCNU-cured mice pretreated with 20 mg/kg of CLZ or 200 mg/kg body weight of STZ did not become susceptible to LSA tumor challenge and the mice survived for
The left footpads received PBS alone. Twenty-four h later, the footpad swelling with 10^7 irradiated (5000 R) LSA or EL-4 tumor cells in their right hind footpads. Growth as described in Fig. 1. On day 10, normal non-tumor-bearing mice. LSA reaction was measured. The footpad swelling for EL-4 has been depicted only for tumor-bearing mice (LSA TBH), LSA tumor-bearing mice treated with CLZ (LSA CLZ), and LSA tumor bears treated with STZ (LSA STZ) were all challenged with 10^6 irradiated (5000 R) LSA or EL-4 tumor cells in their right hind footpads. The left footpads received PBS alone. Twenty four h later the footpad swelling reaction was measured. It was observed (Fig. 5) that normal tumor-bearing mice or tumor-bearing mice treated with STZ failed to elicit DTH response. In contrast, CLZ-treated mice elicited a strong tumor-specific DTH reactivity by selectively depleting tumor-specific suppressor T-cells. LSA tumor-bearing mice were treated as before with 20 mg/kg body weight CLZ or 200 mg/kg body weight of STZ on day 5 post-tumor growth. On day 10, all groups of mice were challenged with 10^6 irradiated LSA or EL-4 tumor cells in their right hind footpads. The left footpads received PBS alone. Twenty four h later the footpad swelling reaction was measured. As demonstrated in Fig. 6, the percentages of CD4+ T-cells in the spleen when compared to untreated controls (Fig. 6). Furthermore, the total number of spleen cells in all the groups tested was not altered significantly (data not shown). Thus, STZ treatment did not affect the absolute numbers of CD4+ T-cells in the spleen when compared to untreated tumor-bearing mice.

CD4+ T-cells in the spleens of tumor-bearing mice and in BCNU- or CLZ-treated tumor-bearing mice were 10, 13.5, and 11.9, respectively. When tumor-bearing mice were treated with 20, 100, or 200 mg/kg body weight of STZ, no significant alterations in the CD4+ T-cell subpopulation were observed, the percentages being 10.3, 9.7, and 9.1 respectively, for these 3 groups (Fig. 6). Furthermore, the total number of spleen cells in all the groups tested was not altered significantly (data not shown). STZ treatment did not affect the absolute numbers of CD4+ T-cells in the spleen when compared to untreated tumor-bearing mice.

DISCUSSION

NUs have been shown to have a broad spectrum of activity against various tumors in vivo (1–5). One of the important characteristics of NUs is their ability to cross the blood-brain barrier and consequently to be useful in the treatment of tumors of the central nervous system. This ability of the NUs to cross the blood-brain barrier is due to the lipophilicity of the drugs. NUs undergo degradation with the formation of a carbonium ion having alkylating properties and organic cyanate with carbamoylating properties. The carbamoylating activity is related to myelosuppression whereas the alkylating property is responsible for tumor cell cytotoxicity (6). In an effort to decrease clinical toxicity while at the same time maintaining therapeutic efficacy, several NUs have been synthesized with altered properties such as water solubility, toxicity, and tissue specificity. In the present study we have used 2 structural analogues of NUs, STZ, and CLZ which have a glucose moiety and low to
no carbamoylating activity and are therefore less myelosuppressive (7, 8). They are also water soluble and are not used in the treatment of brain tumors. In the present study we investigated the therapeutic efficacy of CLZ and STZ and compared it with that of BCNU against LSA tumor syngeneic to C57BL/6 mice.

In our earlier studies, we observed that BCNU treatment of mice bearing a syngeneic tumor, LSA, led to >90% survivors and that these cured mice could reject rechallenge with the homologous tumor but not with a different tumor such as ELO-4 which is also syngeneic to the C57BL/6 mice (9). Furthermore, BCNU treatment of LSA-bearing mice which was highly effective in normal C57BL/6 mice (>90% cures) totally failed when used in nude or irradiated tumor-bearing mice (0% cured) (10). These findings suggested that the immune system, particularly the T-cell component, was essential for successful therapy with BCNU. Based on the above studies we suggested that BCNU treatment may reduce the tumor load, eliminate the T suppressor cells, and thereby permit the effector T-cells to eliminate the tumor cells escaped from the cytotoxic action of the drug. The present study supports this view since 72 h following BCNU or CLZ treatment, significant numbers of tumor cells were still demonstrable in these mice. However, the fact that these mice survived indefinitely in the absence of further drug treatment suggested that the immune system may have been responsible for eliminating the remaining tumor cells.

We have also demonstrated in the LSA tumor system that elimination of tumor-specific T suppressor cells by the drug is critical in the survival of tumor-bearing mice. For example, administration of BCNU at an early stage of tumor growth is not as effective as treatment at an advanced stage of tumor growth, when there is heightened T suppressor cell activity in LSA-bearing mice (9, 11). Following treatment with BCNU, the tumor-specific T suppressor cells were eliminated and such mice could generate heightened cytotoxic T-lymphocyte activity and DTH responses (9, 10). Furthermore, the rejection of the secondary challenge with LSA tumor by BCNU-cured mice was inhibited by adoptive transfer of spleen cells as a source of suppressor cells from normal or LSA tumor-bearing mice (9). These observations together suggested that BCNU eliminates the heightened tumor-specific T suppressor activity present in tumor-bearing mice and that this effect is critical for the cytoxic T-lymphocytes and T helper cells to proliferate and eliminate the tumor cells spared from the action of the drug.

Considering all these possibilities, the failure of STZ even at high doses (200 mg/kg) to cure any LSA tumor-bearing mice can be explained by (a) decreased tumoricidal activity, (b) suppression of T-cell-mediated immunity, or (c) failure to eliminate tumor-specific T suppressor cells. Although STZ at lower doses of 20 or 100 mg/kg was less effective in reducing the tumor load when compared to BCNU or CLZ, at 200 mg/kg STZ had activity comparable to that of BCNU or CLZ. In spite of this, STZ at 200 mg/kg failed to cure tumor-bearing mice thereby ruling out the first possibility. The second possibility, that STZ actively suppressed the host's immunity, can also be ruled out since studies on in vitro T-cell responses to mitogens demonstrated that STZ was indeed less immunosuppressive than BCNU or CLZ. Furthermore, BCNU-cured mice treated with 200 mg/kg of STZ failed to succumb to LSA tumor rechallenge, thereby suggesting that STZ failed to significantly suppress the host's immunity. The third possibility, that STZ failed to eliminate tumor-specific T suppressor cells, appears to be a more likely explanation as to why STZ failed to cure the tumor-bearing mice. For example, it was observed that tumor-bearing mice which failed to elicit tumor-specific DTH response did so when treated with BCNU or CLZ but not following treatment with STZ. The inability of STZ-treated mice to elicit a DTH response was not found to be due to selective elimination by STZ of CD4+ T helper cells which mediate this response (10) as determined by flow cytometric analysis. It was interesting to note that following BCNU or CLZ treatment, the percentage of CD4+ T-cells increased from 10% in untreated tumor-bearing mice to 13.5 and 11.9%, respectively. This increase, although minor, was not seen in STZ-treated mice and therefore may reflect the increased activity of tumor-specific DTH-mediating CD4+ cells in BCNU- and CLZ-treated mice. Since our studies only indirectly suggest that STZ fails to act on suppressor cells, further studies are essential to prove this possibility. For example, can STZ-treated mice generate heightened cytotoxic T-lymphocyte activity similar to BCNU-cured mice (9)? Do STZ-treated mice have enhanced tumor-specific suppressor T-cell activity as demonstrable by cell-mixing experiments and adoptive transfer into BCNU-cured mice (9)? Studies are in progress to address these questions.

Several recent studies have demonstrated that anticancer drugs may enhance immunity of the host and thereby be highly effective in total tumor-eradication. It has been shown earlier that large primary MOPC-315 tumor with metastases can be cured by a single dose treatment with CY (14, 15). This effective cure using CY was due to tumoricidal activity and immunomodulatory activity of the drug which shifted the balance from immunosuppression to immunopotentiation (14, 15). Similarly, injection of bleomycin into rats bearing an advanced stage of KMT-17 fibrosarcoma growth was effective in eliminating suppressor cells which led to an improvement in the therapeutic effects of the drug (16). The fact that effective cancer chemotherapy also depends on the effect of the drug on the immune system is also suggested by the fact that injection of a low dose of CY at a late stage of tumor growth leads to increase in immunity and effectively cures most of the large tumors borne by the host; however, injection of CY at an early stage does not. This may be because CY when administered later eliminates suppressor cells thereby enhancing the host's immunity (17, 18).

In the present study, while comparing the efficacies of different nitrosoureas, we observed that BCNU and CLZ were equally effective in curing advanced LSA tumor growth. This is consistent with earlier studies using mouse L1210 leukemia and LSA tumors wherein it was demonstrated that CLZ had curative antitumor activity comparable to that of BCNU (19, 20). STZ, in contrast, was not effective because it was comparatively less tumoricidal at doses equivalent to those used for BCNU or CLZ. At very high doses (200 mg/kg), although STZ had tumor cytotoxicity comparable to that of BCNU or CLZ, it still failed to cure LSA-bearing mice probably because it failed to eliminate the tumor-specific T suppressor cells and therefore could not convert the immunosuppressive state of the host into an immunodominant stage as seen with BCNU treatment. It would be interesting to study further the alkylating and carbamoylating properties of various nitrosoureas and to determine which of these activities is mainly responsible for elimination of suppressor cells. Although CLZ and STZ have both low carbamoylating properties, since only CLZ can effectively cure LSA-bearing mice, it is likely that the action on T suppressor cells may depend on alkylating rather than carbamoylating properties of the drug.
ACKNOWLEDGMENTS

The authors thank Dr. R. S. Selvan and Aruna Seth for assistance with the research, Carolyn Furrow for secretarial help, and Judith McCord for maintenance of the mouse colony.

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