Involvement of Cytotoxic T-Lymphocytes in the Antitumor Activity of Spergualin against L1210 Cells

Hamao Umezawa, Kiyohiro Nishikawa, Chieko Shibasaki, Katsutoshi Takahashi, Teruya Nakamura, and Tomio Takeuchi

Institute of Microbial Chemistry, 3-14-23, Kamiosaki, Shinagawa-ku, Tokyo 141 [H. U., T. T.]; Research Laboratories, Pharmaceuticals Group, Nippon Kayaku Co., Ltd., 3-31-12, Shimo, Kitakita-ku, Tokyo 115 [K. N., C. S., K. T.]; and Central Research Laboratory, Takara Shuzo Co., Ltd., 3-4-1, Seta, Higashi-ku, Shiga-ken 502-21 [T. N.], Japan

ABSTRACT

Spergualin exhibited a strong antitumor effect against L1210(IMC), a tumor cell line which has been maintained in BALB/c × DBA/2 F1 (hereafter called CD2F1) mice in the Institute of Microbial Chemistry. Mice inoculated i.p. with 106 cells of L1210(IMC) survived more than 60 days by daily i.p. administration of spergualin for 9 days at 5 mg/kg/day, which was started 1 day after the tumor inoculation. These cured mice rejected a second inoculation of 106 cells of L1210(IMC), but they did not reject the inoculation of 103 P388 cells. In Wina's tumor neutralization assay and in the 51Cr release assay, the T-cell fraction prepared from the spleens of the cured mice had higher cytotoxic activity against L1210(IMC) than whole spleen cells. The cytotoxic activity of spleen cells was diminished by treatment with anti-Thy-1.2 or anti-Lyt-2.1 antibody and complement. Therefore, the effector cells involved in the immunological reaction should be regarded as cytotoxic T-lymphocytes. The cytotoxic activity of these T-lymphocytes was measured during and after the spergualin administration for 9 days, and high activity was observed from 1 day after the final spergualin administration. The antitumor effect of spergualin against L1210(IMC) was much lower in T-cell-deficient athymic mice. These results suggest that cytotoxic T-lymphocytes are involved in the antitumor action of spergualin against L1210(IMC) in vivo.

INTRODUCTION

SGL3 is an antitumor antibiotic produced by a strain of Bacillus laterosporus and its structure has been determined to be (−)-(1S)-1-amino-19-guanidino-11,15-dihydroxy-4,9,12-triazanazadecane-10,13-dione (1). SGL exhibited antitumor activity against various mouse leukemias such as L1210, EL-4, and P388 (2, 3). Among SGL-responsive tumors, L1210(IMC) is one of the most sensitive, and the host mice were cured by SGL(7) when inoculated i.p. with 106 cells of L1210(IMC) and the tumor cell line which has been maintained in BALB/c × DBA/2 F1 (hereafter called CD2F1) mice in the Institute of Microbial Chemistry. Mice inoculated i.p. with 106 cells of L1210(IMC) survived more than 60 days by daily i.p. administration of spergualin for 9 days at 5 mg/kg/day, which was started 1 day after the tumor inoculation. These cured mice rejected a second inoculation of 106 cells of L1210(IMC), but they did not reject the inoculation of 103 P388 cells.

MATERIALS AND METHODS

Drugs. SGL was prepared at Takara Shuzo Co., Ltd. (Ohtsu, Japan), dissolved in 0.9% NaCl solution, and stocked in the dark at 4°C before use. ACNU, CYC, and MMC were purchased from commercial sources and dissolved in 0.9% NaCl solution just before use.

Animals. CD2F1, BALB/c, and BALB/c-nu/nu mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan), Charles River Japan Corp. (Atsugi, Japan), and Clea Japan, Inc. (Tokyo, Japan), respectively. T-cell-deficient CD2F1 mice (TXB-CD2F1) were made according to the method of Dye and North (5).

Briefly, CD2F1 mice were thymectomized at 4 weeks of age, and 1 week later the mice were X-ray irradiated (900 rads) followed by i.v. injection with 105 syngeneic bone marrow cells within 2 h after the irradiation. The TXB-CD2F1 mice were used 2 weeks after the irradiation.

Tumor. L1210(IMC) is a strain of L1210 which has been maintained by weekly i.p. transplantation in CD2F1 mice at the Institute of Microbial Chemistry.

Tumor Inoculation. L1210(IMC) cells were collected from the peritoneal cavity with HBSS 5 days after i.p. implantation. After the cell number was determined on a model ZBI Coulter Counter, the cells were diluted with HBSS to the required concentration, and 0.2-ml aliquots of the suspension were inoculated i.p. into the mice.

Drug Administration. SGL was administered i.p. once daily for 9 days starting the next day (day 1) after tumor inoculation. ACNU and CYC were administered i.p. once on day 1. All drugs were administered at a volume of 0.1 ml/10 g body weight.

Preparation of Spleen Cells. Spleens were aseptically collected from mice, and a single cell suspension was prepared by gentle teasing of the spleen mass in HBSS (6). The cell suspension was centrifuged at 200 × g for 5 min, and the cell pellet was resuspended in HBSS or RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. Viability of the cells was measured by the trypan blue dye exclusion method.

Enrichment of T-Cell Population. T-cells were collected from the spleen by the method of Julius et al. (7) with some modifications. Briefly, 2 ml of spleen cell suspension (1.5 to 2.0 × 107 cells) in the supplemented RPMI 1640 were applied onto a nylon wool column equilibrated previously with the culture medium. After incubation at 37°C in a 5% CO2 atmosphere for 45 min, nonadherent cells were collected by elution of the column with 5 ml of the culture medium. The nonadherent cells were washed and resuspended in HBSS for the Winn assay or in the culture medium for 51Cr release assay.

Selective Depletion of Spleen Cell Population by Antibody and Complement. Equal volume of spleen cell suspension (2 × 107/ml) was mixed and incubated with 1:500 diluted anti-Thy-1.2 antibody (Olac 1976, Ltd., Bicester, England), 1:500 diluted anti-Lyt-2.1 antibody (Meiji Institute of Health Science, Tokyo, Japan), 1:5 diluted anti-LT4 antibody (Becton Dickinson, Mountain View, CA), or anti-ascorbate-sensitive antibody solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) at room temperature for 30 min in RPMI 1640 medium. The antibody-treated cells were washed and resuspended in 1:15 diluted rabbit complement solution (Cedarlane Laboratory, Ltd., Hornby, Canada) and incubated further at 37°C for 45 min. The spleen cells thus treated were washed and resuspended in the appropriate assay medium.

Isolation of Macrophage Cells. Harvested peritoneal cells were washed twice with the supplemented RPMI 1640 by centrifugation at 200 × g for 5 min each. Then 4 × 105 viable cells in 2 ml were incubated at 37°C in a 5% CO2 atmosphere for 1 h in plastic dishes (model MSP-P) for macrophage separation (Japan Immunoresearch Laboratory Co., Ltd., Takasaki, Japan). The adherent cells were harvested by washing after incubation at 4°C for 30 min with cold phosphate-buffered saline containing 0.2% EDTA and 5% fetal calf serum. The cells were washed and resuspended in HBSS for the Winn assay.

Received 5/7/86; revised 3/5/87; accepted 3/17/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Deceased.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: SGL, spergualin tribromohydrate; ACNU, 1-(4-aminoo-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosoureio hydrochloride; CYC, cyclophosphamide; MMC, mitomycin C; HBSS, Hanks' balanced salt solution; CTL, cytotoxic T-lymphocytes; TXB, thymectomized, X-ray irradiated, and bone marrow cell-transferred mice.
In Vivo Tumor Neutralization Assay (Winn Assay). A modified Winn assay (8) was done as follows. Immune cells (2 x 10^5) obtained from the cured mice and L1210(IMC) cells (1 x 10^5) were mixed and incubated in 0.2 ml HBSS for 15 min at 37°C and then inoculated i.p. into CD2F1 mice. The neutralizing effect of immune cells was evaluated by mean survival time of the inoculated mice. Mice in a control group were inoculated with L1210(IMC) cells alone.

Cr Release Assay. Spleen cells were stimulated in vitro by a 5-day coculture with L1210(IMC) cells pretreated with MMC (50 μg/ml for 1 h) (9). Cytolytic activity of spleen cells was determined by Cr release by mean survival time of the inoculated mice. Mice in a control group into CD2F1 mice. The neutralizing effect of immune cells was evaluated incubated in 0.2 ml HBSS for 15 min at 37°C and then inoculated for 4 h with 4 cycles with the culture medium. Effector cells and the labeled target cells (2 x 10^6) in 0.15 ml of the culture medium were mixed and incubated for 4 h at 37°C in a CO2 incubator, and thereafter the radioactivity of the supernatant was measured with a Beckman model Gamma 8000 gamma spectrometer. The cytosis was calculated as

\[
\text{Cytolysis} = \frac{\text{Test release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100
\]

Maximum release of Cr was obtained by 3 repetitive freezings and thawings of the labeled L1210(IMC) cells.

RESULTS

Eighty-four % or more of L1210(IMC)-bearing mice survived for 45 days after the inoculation when SGL, ACNU, and CYC were separately administered under the dose schedules shown in Table 1. The surviving mice were reinoculated with various numbers of L1210(IMC) cells and their survival times were examined. The mice cured by SGL rejected the growth of the second inoculum of L1210(IMC) (up to 10^6 cells), whereas those cured by ACNU or CYC did not reject the second one even when its size was reduced to 10^5 cells. The mice cured by SGL did not reject P388 cells, even when the inoculum size was only 10^5 cells, although the mean survival times of the cured mice challenged with P388 were slightly longer than those of the control mice. Thus, the rejection of the second inoculation was found to be specific to L1210(IMC) cells. This specific rejection was also observed when L1210(IMC) cells were reinoculated s.c. (data not shown). The neutralizing effect of immune cells was evaluated by mean survival time of the inoculated mice. Mice in a control group were inoculated with L1210(IMC) cells alone.

To characterize the effector cells responsible for the tumor rejection, we carried out Cr release assays. As shown in Table 3, lysis of Cr-labeled L1210(IMC) cells was observed after 4 h incubation with the spleen cells which were isolated from the mice cured from L1210(IMC) by SGL and previously stimulated by coculture with MMC-treated L1210(IMC) cells for 5 days. These stimulated spleen cells did not show any cytolytic activity to Cr-labeled P388 and YAC-1 cells. The in vitro stimulation of the spleen cells with the MMC-treated L1210(IMC) cells was necessary to induce cytolytic activity. Moreover, when the MMC-treated stimulator cells were replaced with MMC-treated P388 cells, cytolytic activity was not induced to Cr-labeled P388 cells and to L1210(IMC) cells. These results suggest that the cytolytic activity of spleen cells from the mice cured by SGL is specific to L1210(IMC) cells and responsible for in vivo tumor rejection.

For further characterization of effector cells, some treatments on spleen cells were performed before coculture with Cr-

Table 1 Rejection of second tumor inoculation in L1210(IMC) mice surviving by treatment with SGL, ACNU, and CYC

<table>
<thead>
<tr>
<th>Drug and Dose (mg/kg x day)</th>
<th>Survivor rate*</th>
<th>Second inoculation</th>
<th>Tumor</th>
<th>MST^</th>
<th>Survival rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control mice</td>
<td>99/118 (84%)</td>
<td>L1210(IMC)</td>
<td>10^7</td>
<td>5.2 ± 0.4</td>
<td>0/5</td>
</tr>
<tr>
<td>SGL, 5 x 9</td>
<td>29/30 (97%)</td>
<td>L1210(IMC)</td>
<td>10^7</td>
<td>6.0 ± 0.7</td>
<td>0/5</td>
</tr>
<tr>
<td>ACNU, 30 x 1</td>
<td>55/60 (92%)</td>
<td>L1210(IMC)</td>
<td>10^7</td>
<td>10.0 ± 0.0</td>
<td>0/6</td>
</tr>
<tr>
<td>CYC, 300 x 1</td>
<td>99/118 (84%)</td>
<td>P388</td>
<td>10^7</td>
<td>12.2 ± 1.3</td>
<td>0/6</td>
</tr>
</tbody>
</table>

*Forty-five days after the first inoculation of 10^6 L1210(IMC) cells.
^MST, mean survival time (mean ± SD).
*Fifty days after the second inoculation.
*First inoculation and treatment were not performed.

Table 2 Characterization of effector cells by Winn assay

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>Survival time</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Control)</td>
<td>9.2 ± 0.8*</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Spleen cells</td>
<td>14.7 ± 5.8</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>Spleen cells</td>
<td>19.5 ± 7.0</td>
<td>212</td>
<td></td>
</tr>
<tr>
<td>Spleen cells</td>
<td>8.7 ± 0.5</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Spleen cells</td>
<td>8.8 ± 0.5</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Peritoneal cells</td>
<td>11.0 ± 1.3</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>

*Spleen cells and peritoneal cells were harvested from the mice cured of L1210(IMC) by SGL 45 days after inoculation. Treatments were performed as described in "Materials and Methods." L1210(IMC) cells (10^6) were incubated separately with each population of immune cells (2 x 10^6) for 15 min and then inoculated i.p. into CD2F1 mice.

Table 3 Specificity of cytolytic activity of spleen cells from L1210(IMC)-implanted mice treated with SGL

<table>
<thead>
<tr>
<th>Stimulator cells*</th>
<th>Target cells^</th>
<th>Cytolysis (%) at effector:target cell ratio of</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>L1210(IMC)</td>
<td>0</td>
</tr>
<tr>
<td>L1210(IMC)</td>
<td>P388</td>
<td>36 ± 48</td>
</tr>
<tr>
<td>P388</td>
<td>YAC-1</td>
<td>0</td>
</tr>
</tbody>
</table>

*Spleen cells from SGL-cured mice were cocultured with MMC-treated stimulator cells for 5 days.
^Cr-labeled target cells were mixed and incubated with stimulated spleen cells for 4 h. See "Materials and Methods" for details.
labeled L1210(IMC) cells (Table 4). The T-cell fraction prepared by passage through a nylon wool column showed the stronger cytolytic activity than whole spleen cells. The cytotoxicity was diminished by treatment of spleen cells with anti-Thy-1.2 antibody or anti-Lyt-2.1 and complement. Treatment of spleen cells with anti-L3T4 antibody or anti-asialo-GM1 antibody and complement did not affect the activity of spleen cells. These results suggest that the effector cells induced by SGL treatment in vivo are CTL. However, when the spleen cells were first fractionated and the T-cell fraction were cocultured with MMC-treated L1210(IMC) cells, then the T-cells did not show cytolytic activity. These results suggest that the simultaneous presence of other type(s) of immune cells is necessary for CTL induction.

Next, we determined when the cytolytic activity of the spleen cells is induced by SGL in L1210(IMC)-implanted mice. When SGL at 5 mg/kg/day was administered daily for 9 days for 9 days starting the day (day 1) after the tumor inoculation, the cytolytic activity of the spleen cells appeared on day 9 and became maximal on day 13 (Table 5). However, the cytotoxic activity induced by the administration of 15.8 mg/kg/day of SGL was small and temporary, and a subtoxic dose (50 mg/kg/day) of SGL did not induce CTL at all. When a much lower dose (1.58 mg/kg/day) of SGL was administered, higher cytolytic activity was induced on day 9, although after that mice died from tumor.

We examined the effect of the reinoculation time on the rejection of L1210(IMC) cells. As shown in Table 6, the mice treated with SGL at 5 mg/kg/day for 9 days rejected 10^6 L1210(IMC) cells even when the reinoculation was done on day 10. This result is consistent with the fact that CTL have been already induced on day 10, the day after the final injection of SGL at a dose of 5 mg/kg/day.

In order to confirm the involvement of immunological action in the antitumor effect of SGL, the therapeutic effect of SGL against L1210(IMC) was compared between normal and T-cell-deficient athymic mice. As shown in Fig. 1A, the increase in life span in TXB-CD2F, mice was much lower than that in normal CD2F, mice at lower doses of SGL (3.13 to 12.5 mg/kg/day). At a high dose of SGL, 50 mg/kg/day, where the CTL was not induced, the increase in life span of normal mice was decreased, almost being the same as that of TXB-CD2F, mice. These results suggest that CTL activity induced by low doses of SGL in normal mice contributes to the prolongation of life in such treated animals.

Therapeutic effect of SGL in athymic nude BALB/c mice was also much lower than that in normal BALB/c mice (Fig. 1B). In this case, however, all BALB/c mice survived at any dose of SGL used. Although BALB/c and L1210 (DBA/2 in origin) carry the same H-2d haplotype, the effector cells induced in BALB/c mice seemed to be alloreactive CTL because the spleen cells from the cured BALB/c mice caused cytolysis of P388 cells (H-2k, DBA/2 in origin) as well as L1210(IMC) cells but not of MH134 cells (H-2d, C3H/He in origin) in the \textsuperscript{51}Cr release assay (data not shown). Dose-response patterns in nude mice and in TXB mice were similar to each other. This weak antitumor activity shown by higher doses of SGL in the two kinds of T-cell-deficient athymic mice may be due to the direct antitumor action of SGL on L1210(IMC) cells.

**DISCUSSION**

When L1210(IMC)-bearing mice were cured (i.e., surviving more than 45 days) by treatment of SGL at the low dose, these mice rejected a second inoculation of 10^6 L1210(IMC) cells. This immunological resistance was shown to be due to the induction of CTL. In both the Winn assay and \textsuperscript{51}Cr release assay the T-cell fraction had higher activity than the whole...
These results may be related to the direct stimulation of the
by SGL in the stimulation process in vitro with MMC-treated
L1210(IMC) cells before the $^{51}$Cr release assay was conducted.
These results may be related to the direct stimulation of the
induction of CTL by macrophages and dendritic cells or to the
indirect stimulation through soluble mediators (11, 12). Al-
though further cellular mechanisms involved in the induction
of CTL remain unclear, enhancement of production of IL-2 by
SGL seemed to be involved (13).

Although a lower dose of SGL (5 mg/kg/day) to
L1210(IMC)-bearing mice induced CTL, a 10-fold higher dose
(50 mg/kg/day) did not. This result is in accord with the fact
that SGL exhibits its antitumor activity against L1210(IMC)
implanted into CD2F1 mice which cannot induce CTL and may be related to the
reported immunosuppressive effect of high doses of SGL on
the establishment of delayed-type hypersensitivity to sheep
RBC (4).

The antitumor activity of SGL observed in thymectomized
mice may be due to direct antitumor action of SGL. The
immunological rejection and the induction of CTL occurred 1
day after the final administration of 9 consecutive daily doses
at 5 mg/kg/day. These results will be interpreted to mean that
direct antitumor action of SGL reduced the number of tumor
cells less than $10^6$ and then CTL removed the residual tumor
cells.

There are a few reports on rejection of the second tumor
inoculation in mice cured by other antitumor agents such as
1,3-bis(2-chloroethyl)-1-nitrosourea (14), CYC (15) and bleo-
mycin (16). In our present study the mice cured of L1210(IMC)
by ACNU, an analogue of 1,3-bis(2-chloroethyl)-1-nitrosourea,
or by CYC did not reject rechallenged L1210(IMC) cells, even
when the challenged dose was as low as $10^5$ cells.

From the results presented in this paper, we can conclude
that SGL exhibits its antitumor activity against L1210(IMC)
by induction of CTL in addition to its direct antitumor action.

REFERENCES

1. Umezawa, H., Kondo, S., Linuma, H., Kunimoto, S., Ikeda, Y., Iwasaki, H.,
2. Takeuchi, T., Linuma, H., Kunimoto, S., Masuda, T., Ishizuka, M., Takeuchi,
M., Hamada, M., Nagaawara, H., Kondo, S., and Umezawa, H. A new antitumor
3. Nishikawa, K., Shibasaki, C., Takahashi, K., Nakamura, T., Takeuchi, T.,
and Umezawa, H. Antitumor activity of spergualin, a novel antitumor anti-
4. Umezawa, H. Studies of microbial products in rising to the challenge of
5. Dye, E. S., and North, R. J. T cell-mediated immunosuppression as an
adoptive immunotherapy of the P815 mastocytoma and its metastases. J.
7. Julius, M. H., Simpson, E., and Herzenberg, L. A. A rapid method for the
isolation of functional thymus-derived murine lymphocytes. Eur. J. Immu-
8. Winn, H. J. Immune mechanisms in homotransplantation II. Quantitative
 assay of the immunologic activity of lymphoid cells stimulated by tumor
9. Mokyr, M. B., and Dray, S. In vivo immunization as a method for generating
cytotoxic cells potentially useful in adoptive immunotherapy. Methods Can-
immunity in mice I. Induction, development and in vitro assay of cellular
lines III. Accessory cell requirements for the growth of cloned cytolytic T
12. Nussenzweig, M. C., Steinman, R. M., Gutchikov, B., and Cohn, Z. A.
Dendritic cells are accessory cells for the development of anti-trinitrophhenyl
13. Ishizuka, M., Masuda, T., Mizutani, S., Ono, M., Kumagai, H., Takeuchi,
T., and Umezawa, H. Induction of antitumor resistance to mouse leukemia
14. Feola, J., and Maruyama, Y. Host resistance to tumor after cure of advanced
LSA lymphoma by treatment with BCNU and chlorozotocin. Cancer Treat.
15. Hengst, J. C. D., Mokyr, M. B., and Dray, S. Cooperation between cyclo-
phosphamide tumoricidal activity and host antitumor immunity in the cure
H. Host-mediated therapeutic effects produced by appropriately timed
Involvement of Cytotoxic T-Lymphocytes in the Antitumor Activity of Spergualin against L1210 Cells

Hamao Umezawa, Kiyohiro Nishikawa, Chieko Shibasaki, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/12/3062

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/47/12/3062. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.