Changes in Cellular Components of Spleen and Lymph Node Cells and the Effector Cells Responsible for Meth A Tumor Eradication Induced by Zinostatin Stimalamer

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ABSTRACT

We reported previously that pretreatment with zinostatin stimalamer (ZSS) eradicated Meth A tumors in BALB/c mice. We herein investigated cellular components of spleen and lymph node cells of Meth A-bearing ZSS-pretreated mice by flow cytometry; the antitumor effector cells by in vivo depletion of T cells, NK cells, or macrophages; and host-mediated antitumor activity associated with ZSS treatment after tumor transplantation. ZSS given on day -3 transiently decreased the number of spleen cells. The percentage of T cells increased, but B cells and macrophages decreased. B cells decreased in inguinal lymph nodes in Meth A-bearing ZSS-pretreated mice, but increased in Meth A-bearing control mice. In vivo depletion experiments using antibodies or carrageenan showed that antitumor effector cells for tumor eradication are Thy1.2+/Lyt2.2+ and that at least a part of them are asialo GM1+. Thy1.2+/Lyt2.2+/asialo GM1+ cells are important in generation of the antitumor activity of ZSS; however, L3T4+ T cells are also involved in initiation of tumor eradication. The result of ZSS treatment after tumor transplantation suggests that ZSS might exhibit antitumor activity by augmenting host-mediated antitumor resistance, as well as its intrinsic cytotoxic activity.

INTRODUCTION

ZSS3 is an anticancer agent synthesized by conjugation of one molecule of neocarcinostatin and two molecules of poly(styrene-co-maleic acid) (2-4). In our previous paper (1), we reported that treatment with ZSS before Meth A transplantation induced tumor eradication after transient tumor growth. Single injection of ZSS between 1 day and 4 weeks before tumor transplantation induced tumor eradication. Tumor regression started from about 10 days after tumor transplantation, and splenic T cells from the mice showed tumor-neutralizing activity by the Winn assay (5). Because Apo-ZSS, which lacks cytotoxic activity, did not induce tumor regression, the cytotoxic activity of ZSS is essential for induction of the host-mediated antitumor effect (1).

To investigate the mechanism of tumor eradication induced by ZSS pretreatment in more detail, we first examined the effect of ZSS pretreatment on spleen and lymph node cells by flow cytometric analysis, especially on T cells using anti-L3T4 and anti-Lyt2 mAb (6-8), NK cells using anti-asGM1 (9), and macrophages using anti-Mac-1 mAb (10), which were suggested to be antitumor effector cells in various tumor models. Second, we attempted to identify the antitumor effector cells responsible for tumor eradication and their possible precursor cells in the early phase by in vivo depletion using antibodies against various surface antigens of T lymphocytes or carrageenan in Meth A-bearing ZSS-pretreated mice. Finally, we studied in vivo T-cell depletion to investigate whether host-mediated antitumor resistance is involved in the therapeutic effect of ZSS.

MATERIALS AND METHODS

Animals and Tumor Cells. Female BALB/c mice 6-9 weeks old were purchased from Japan SLC (Shizuoka, Japan). Meth A, a methylcholanthrene-induced fibrosarcoma cell line, was maintained by i.p. passages in BALB/c mice. Meth A cells (5 × 10^6 to 1 × 10^7 cells/mouse) were transplanted into BALB/c mice s.c. at the flank (the day of transplantation is defined as day 0). The animals were treated in compliance with institutional guidelines.

Drug. ZSS (SMANCS, Yamanouchi Pharmaceutical, Tokyo) was dissolved in sterile 0.01 M PBS just before use. ZSS was administered once i.v. to mice 3 days or 3 weeks before Meth A transplantation. In therapeutic treatment (posttreatment), ZSS was administered 3 days after tumor transplantation. Preparation and i.v. administration of the drug were performed under dim lights to prevent light inactivation.

Antibody. FITC-conjugated anti-Lyt2 mAb (clone 53-6-7), PE-conjugated anti-L3T4 mAb (clone GK1.5), FITC-conjugated goat antimouse immunoglobulin and PE-conjugated rat antimouse α chain mAb were purchased from Becton Dickinson (Mountain View, CA). Antimouse macrophage mAb (anti-Mac-1, clone M1/70.15.1) and FITC-conjugated P(ab')2 of rabbit antirat immunoglobulin were purchased from Serotec (Oxford, England). Anti-Thy1.2 mAb, rabbit anti-asGM1, and FITC-conjugated goat antirabbit IgG were purchased from Cedarlane ( Hornby, Ontario, Canada), Wako Pure Chemical Industries (Osaka, Japan), and Medical and Biological Laboratories (Nagoya, Japan), respectively.

Anti-L3T4 hybridoma (GK1.5 cells) and anti-Lyt2 hybridoma (2.43 cells) were purchased from the American Type Culture Collection (Rockville, MD) and were cultured in DMEM with 4.5 g/L glucose containing 20% fetal bovine serum. Cells were inoculated i.p. into BALB/c nu/nu mice and their ascites was harvested. Fifty % ammonium sulfate precipitates of ascites were well diaлизed with PBS and used as anti-L3T4 or anti-Lyt2.2 mAb for the in vivo depletion test.

Preparation of Spleen and Lymph Node Cells and Examination of Cellular Components. ZSS was administered to BALB/c mice 3 days before Meth A transplantation (2.5 mg/kg). Spleens and inguinal lymph nodes were harvested before and after treatment, and spleen cells and lymph node cells were prepared as described previously (11). Briefly, the cells were flushed out using forceps into RPMI 1640, and spleen cells were additionally treated with Tris-buffered ammonium chloride (0.83%) to remove erythrocytes. Viable cells were counted by the trypan blue exclusion method, and the cells were stained for flow cytometric analysis.

The number of splenic T cells was calculated by multiplying the number of total spleen cells by the sum of the percentages of L3T4+ cells, Lyt2+ cells, and L3T4+/Lyt2+ cells. The number of other spleen and lymph node cells were also calculated by multiplying the total number of cells by the percentages of each population.

Flow Cytometry. The cells were stained by direct or indirect immunofluorescence. In the case of direct staining, 2 × 10^6 to 5 × 10^6 spleen cells or lymph node cells were suspended in PBS, treated with fluorescein-conjugated antibody for 20 min on ice, and then suspended in 0.5 ml PBS after washing with PBS. In the case of indirect staining, the cells were treated with primary antibody, and after washing in PBS they were treated with fluorescein-conjugated secondary antibody. Staining with anti-Mac-1 mAb or anti-asGM1 was performed by an indirect staining method, and staining with other fluo...
rescein-conjugated mAb or antimouse immunoglobulin was performed by a direct staining method. Two-color staining was performed with FITC-conjugated anti-L3T4 mAb and PE-conjugated anti-Lyt2 mAb. The optimal concentrations of these antibodies for staining were determined in a preliminary experiment. Nonspecific binding of FITC-rabbit antirat immunoglobulin to mouse surface immunoglobulin on B cells was reduced by the addition of normal mouse serum. More than $5 \times 10^8$ cells were analyzed using an Epics Profile (Coulter Electronics, Hialeah, FL). Unstained cells and cells stained with only secondary antibody were also analyzed as a control.

In Vivo Depletion Test. ZSS was administered to BALB/c mice either 3 days (2.5 mg/kg) or 3 weeks (1 mg/kg) before Meth A transplantation. For in vivo depletion of T cells, 40 µl anti-Thy.1.2 mAb diluted to 0.4 ml with saline was injected i.v. Flow cytometric analysis 2 days after injection showed that splenic Thy.1.2+ cells were abolished in the mice. For the in vivo depletion of asialo GM1+ cells, 50 µl anti-asGM1 diluted to 0.2 ml with saline were injected i.v. By this treatment, asialo GM1+ cells were deleted completely and the proportion of Mac-1+ spleen cells was slightly decreased. Because Mac-1 antigen is known to be expressed not only on macrophages/monocytes but also on granulocytes and NK cells (10, 12), it is possible that the Mac-1+ cells depleted by anti-asGM1 treatment were NK cells. Carrageenan (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.85% NaCl at the concentration of 3.5 mg/ml and was autoclaved. Carrageenan (0.7 mg) was injected i.p. to deplete phagocytes (13). To examine the role of T lymphocyte subsets, anti-L3T4 mAb 1 mg or anti-Lyt2.2 mAb 1 mg in 0.2 ml saline were injected i.v. into mice to deplete CD4+ cells or CD8+ cells, respectively. By treatment with each antibody, L3T4+ and Lyt2+ cells were depleted completely in spleen cells, and the depletion continued for at least 7 days after a single injection. No apparent change was observed in the other subsets. Tumor diameter was measured using calipers, and tumor volume was approximated using the following formula (14):

\[
\text{Tumor volume (cm}^3) = \frac{\text{short diameter (mm)}^2 \times \text{long diameter (mm)}}{2}
\]

Percent of inhibition was calculated using the following formula from the mean tumor volume of test mice and that of control mice.

\[
\% \text{ inhibition} = 1 - \frac{\text{mean of test}}{\text{mean of control}} \times 100
\]

Host-mediated Antitumor Activity Induced by Therapeutic Treatment with ZSS. BALB/c mice were given 2.5 mg/kg of ZSS 3 days after Meth A transplantation. Anti-Thy.1.2 mAb (50 µl diluted to 0.5 ml with saline) was injected i.v. on days 10 and 13. Tumor diameters were measured, and tumor volume was approximated as described above.

Statistical Analysis. Statistical analysis of the data was performed by Student's t test. P < 0.05 was taken as the level of significance.

RESULTS

Changes in Spleen Cell Population during Meth A Eradication. When spleen cells were examined every 7 days after tumor transplantation, the total number of spleen cells from Meth A-bearing ZSS-pretreated mice (ZSS-pretreated mice) decreased only on day 7 to about 70% of that of tumor-bearing control mice (tumor control mice) and normal mice, and it recovered almost to the level of normal mice by day 14 (Table 1). As shown in Fig. 1D, changes in the cell population were only seen on day 7. The percentages of L3T4+ cells and Lyt2+ cells were increased significantly (143% and 130% of mean percentage of normal mice, respectively) but the percentage of Mac-1+ cells was decreased (35% of mean percentage of normal mice). No significant changes were observed in the percentage of asialo GM1+ cells because of rather large individual differences even in normal mice (Table 2). In tumor control mice, a slight decrease of Lyt2+ cells and a slight increase of Mac-1+ cells were observed on day 20 (Fig. 1C).

Focusing on the change observed on day 7, the spleen cell population was further examined on days 0, 1, 3, and 6 after tumor transplantation. In this experiment, B cells were also examined as slg-positive cells. As shown in Fig. 2A, the total number of spleen cells decreased remarkably after ZSS treatment, and the nadir occurred on day 0 (3 days after ZSS administration). The percentages of spleen cell subpopulations are shown in Fig. 1B. The percentages of L3T4+ cells and Lyt2+ cells were significantly increased in ZSS-pretreated mice and reached 168% and 163%, respectively, of normal mice on day 0. In contrast with these findings, the percentages of B cells and Mac-1+ cells significantly decreased from day 0 to 6.

| Table 1 Total number of splenocytes from ZSS-pretreated mice and control mice |
|-------------------|-------------------|-------------------|-------------------|
| Experiment | Normal | Tumor | ZSS* | Normal | Tumor | ZSS | Normal | Tumor | ZSS |
| 1 | 6.7* | NT* | 8.2 ± 0.5* | 10.9 ± 1.4 | 10.0 ± 0.7 | 11.5 ± 1.0 | 10.8 ± 0.8 | 9.0 ± 1.0 |
| 2 | 7.0 | 4.5 | 5.6 | 8.7 | 6.6 | 6.9 | 11.2 | 5.3 |

* Normal, normal mice; tumor, Meth A-bearing mice; ZSS, Meth A-bearing mice pretreated with 2.5 mg/kg of ZSS on day −3.
NT, not tested.
Mean ± SE (×10^5 cells/mouse) of 9–13 mice (pooled splenocytes were counted).
Asterisks, significant differences between the tumor control mice and ZSS-pretreated mice. Mice were administered 2.5 mg/kg ZSS or nothing on day -3. Changes in the number of splenic T cells and B cells in ZSS-pretreated mice are shown in Fig. 2, B and C. Depletion of B cells was not observed (Fig. 2B). The effect of treatment with these antibodies or carrageenan was the same when the mice were pretreated with 1 mg/kg ZSS 21 days before tumor transplantation (Table 3).

Effect of Treatment with Antibodies and Carrageenan on Induction Phase of Antitumor Activity by ZSS Pretreatment. Anti-Thyl.2 mAb was injected before and after transplantation (days -2 and 1), and their effect on the antitumor activity of ZSS pretreatment was compared with that of treatment before tumor regression (days 3 and 6) or with that of treatment after the start of tumor regression (days 10 and 13). The results are shown in Fig. 5. Tumors were eradicated in 4 of 5 ZSS-pretreated mice, whereas in all mice treated with anti-Thyl.2 mAb, tumor eradication was completely inhibited. The tumor volume of 2 of 4 mice treated with anti-Thyl.2 mAb on days -2 and 1 was increased more than that of tumor-bearing control mice. Additionally, the later the antibody was administered, the smaller the inhibitory effect of anti-Thyl.2 mAb on tumor regression. The effect of a single injection of anti-Thyl.2 mAb 1 day before transplantation was enough to prevent tumor regression induced by ZSS until day 21 (data not shown). Neither treatment of anti-asGM1 before and after transplantation (days -2 and 1) nor that of carrageenan before transplantation (day -1) had any effect on the antitumor activity induced by ZSS pretreatment (data not shown).

Identification of Effector T-Cell Subset Responsible for Antitumor Activity of ZSS. Mice were given anti-L3T4 or anti-Lyt2 mAb on day -2, 7, or 14 after tumor transplantation. In the mice treated with anti-L3T4 mAb, significant inhibition of tumor regression was not observed and the tumor was eventually eradicated in all mice.
Table 3 Effector cells induced by pretreatment with ZSS 3 weeks before tumor transplantation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ZSS</th>
<th>Antibody or carrageenan</th>
<th>n</th>
<th>mean ± SE inhibition (%)</th>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>8</td>
<td>3.51 ± 0.44</td>
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<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>9</td>
<td>0.08 ± 0.08</td>
</tr>
<tr>
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<td>anti-Thy1.2 mAb (days 10 and 14)</td>
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<td>3.75 ± 0.72</td>
<td>−6.8</td>
</tr>
<tr>
<td>+</td>
<td>anti-asGM1 (days 7, 10, and 14)</td>
<td>5</td>
<td>1.92 ± 0.98</td>
<td>45.3</td>
</tr>
<tr>
<td>+</td>
<td>carrageenan (days 7, 10, and 14)</td>
<td>7</td>
<td>0.03 ± 0.02</td>
<td>99.2</td>
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* Mice were given 1 mg/kg of ZSS on day −21 and treated with or without anti-Thy1.2 mAb i.v., anti-asGM1 i.v., or carrageenan i.p. on indicated days.

** Tumor diameters were measured on day 28.

** P < 0.05 (compared to ZSS-pretreated mice).

*** P < 0.001 (compared to ZSS-pretreated mice).

Fig. 4. Effect of treatment of anti-Thy1.2 mAb, anti-asGM1, and carrageenan on antitumor activity induced by pretreatment with ZSS. Mice were given 2.5 mg/kg ZSS on day −3 and treated with anti-Thy1.2 mAb i.v. (Ø; A), anti-asGM1 i.v. (Δ; B), carrageenan i.p. (□; C), or were untreated (○; ZSS control; A and B) on days 7, 10, and 14. Tumor control mice were given Meth A transplantation only (○). Each value represents the mean tumor volume of 3–5 mice in each group; bars, SE. Differences between anti-Thy1.2 mAb-treated mice and ZSS control mice were statistically significant at P < 0.05 (day 14) and P < 0.01 (day 21, day 28). Differences between anti-asGM1-treated mice and ZSS control mice were statistically significant at P < 0.01 (day 14) or P < 0.001 (days 17, 21, and 31).

DISCUSSION

As described previously, after transient growth, Meth A tumor regression was observed from day 10. From day 14, tumor-neutralizing activity was shown in the spleen cells from the mice and the effector cells were T cells (1). We examined the population of spleen cells having tumor neutralizing activity; however, both the cell population containing T-cell subsets and the total number of spleen cells did not change during tumor regression (Table 1; Fig. 1). In normal BALB/c mice, spleen cells were composed of about 40% T cells, 45% B cells, and 15% monocytes/macrophages.

(Fig. 6A). Conversely, Meth A eradication was completely prevented by anti-Lyt2.2 mAb in all mice regardless of the timing of treatment, and the effect of anti-Lyt2.2 mAb lasted for more than 1 week after injection. The effect of anti-Lyt2.2 mAb treatment was more prominent when injected earlier (Fig. 6B).

When anti-Lyt2.2 and anti-L3T4 mAbs were injected simultaneously before tumor transplantation, anti-L3T4 mAb showed an additive effect in comparison with a single injection of anti-Lyt2.2 mAb (Fig. 7).

Effect of Therapeutic Treatment of ZSS on Host-mediated Antitumor Activity. ZSS was administered 3 days after tumor transplantation and anti-Thy1.2 mAb was injected on days 10 and 13. As shown in Fig. 8, the therapeutic administration of ZSS was also effective, as in the case of pretreatment, and tumors were eradicated in 5 of 6 mice. However, tumors were not eradicated in any of the mice treated with anti-Thy1.2 mAb, and tumor growth was partially reversed.
B cells, 5–10% Mac-1+ cells, and 5–20% asialo GM1+ cells. Evident changes in the spleen were observed in the induction phase of antitumor activity as follows. After the administration of ZSS on day -3, T cells increased up to 76.3% without a concomitant change in the CD4/CD8 (L3T4/Lyt2) ratio, and B cells and Mac-1+ cells decreased by 19.8% and 3.5%, respectively (Fig. 1). The effect of ZSS on B cells was the same in lymph nodes (Fig. 3). A similar effect on B cells was reportedly induced by cyclophosphamide, which has the ability to induce host-mediated antitumor activity (15, 16). Inguinal lymph nodes of tumor control mice were enlarged on day 21, together with a clear increase in B cells (Fig. 3A). It is notable that B cells, which were decreased by ZSS treatment, increased in Meth A-bearing mice; however, antibody formation against tumor cell antigen, which is reported to be an escape mechanism of tumor cells from immunological control (17), might explain this finding. Cyclophosphamide and cytostine arabinoside reportedly suppress antibody formation by affecting B cells specifically and intensify antitumor resistance as a result (18, 19). Antitumor necrosis factor antibody production might be involved in tumor-escape mechanisms (20). It is thought to be beneficial for tumor-bearing hosts to inhibit production of neutralizing antibodies against cytokines having antitumor activity. In our tumor model, it should be further investigated whether the decrease in B cells is involved in the antitumor effect induced by ZSS.

Meth A was eradicated by ZSS pretreatment 3 or 4 weeks before tumor transplantation (1). Although the changes described above were evident on the day of tumor transplantation in the mice pretreated on day -3, no significant change was observed on the day of transplantation when ZSS was administered on day -21 (data not shown). These results suggest that the change in the cell population at the time of tumor transplantation was not required for induction of ZSS-mediated antitumor activity. Although the cytotoxic activity of ZSS was essential to induce host-mediated antitumor activity (1), no significant cytotoxic activity was detected in vivo 24 h after i.v. injection of ZSS (21). These facts suggest that ZSS might affect the precursor cells involved in tumor eradication through its cytotoxicity, and that the effect lasts for a long time.

As summarized in Table 4 on in vivo depletion test, Thy1.2+/Lyt2.2+ T cells are suggested to be the effector cells responsible for tumor eradication by the treatment with antibodies or carrageenan (Table 4, I, II, and IV). Anti-asGM1, which is used for NK cell depletion, also prevented tumor eradication, but some antitumor activity remained (Table 4, I and II). On the basis of these, it was suggested that asialo GM1+ antitumor effector cells are not NK cells and that at least some Thy1.2+ antitumor effector cells might express asialo GM1 antigen as well. This is supported by the fact that NK activity was not augmented in splenocytes from ZSS-pretreated mice (1). It is known that asialo GM1 is expressed not only on NK cells but also on activated macrophages and activated T cells (22–24). The functions of asialo GM1+ T cells were investigated in various laboratories as follows. A small population of adult murine thymocytes, which express asialo GM1, have CTL precursor activity, proliferative capacity and interleukin-2 production (25). The majority of alloimmune CTLs and some CTL clones express asialo GM1 (26, 27). Cytolytic cells generated in F1 mice injected with parental spleen cells
are asialo GM1/Thy1.2/Lyt2.2 (28). Effector cells of the tumor-neutralizing activity of spleen cells from MOPC104E tumor-bearing mice treated with interleukin-1 and a low dose of cyclophosphamide are suggested to be asialo GM1/Lyt2.2/Thy1.2 (29). Such an asialo GM1 T-cell subpopulation is thought to be the effector cells involved in Meth A eradication induced by ZSS. The results of treatment with antibodies or carrageenan before and after transplantation suggest that asialo GM1/Lyt2.2/Thy1.2 T-cells are important for the initiation of Meth A eradication, and that antitumor activity is induced without the help of either asialo GM1 cells or macrophages (Table 4, III-V). The results of simultaneous injection of anti-Lyt2 and anti-L3T4 mAbs before tumor transplantation suggests that L3T4+ T-cells are also involved in the generation of antitumor effector cells (Table 4, VI). From these results, it was suggested that asialo GM1/Lyt2.2/Thy1.2 T-cells are activated by ZSS pretreatment and tumor transplantation, which was essential for induction of antitumor activity (1), and thereafter asialo GM1/Lyt2.2/Thy1.2+ (and possibly asialo GM1/Lyt2.2/Thy1.2+) antitumor effector cells that eradicated Meth A tumor were generated.

When ZSS pretreatment was performed 3 weeks before tumor transplantation, the induced effector cells were of the same phenotype as those that appeared in mice pretreated with ZSS on day −3 (Table 4, I and II). This suggests that the mechanism of tumor eradication is the same regardless of the interval between pretreatment with ZSS and tumor transplantation.

Because ZSS has potent cytotoxic activity to various tumor cells (4) involving DNA degradation and inhibition of DNA synthesis (30), it had been thought that its antitumor activity was mainly due to its direct cytotoxic activity against tumor cells when ZSS was administered after tumor transplantation. Therefore, the effect of ZSS on host-mediated antitumor activity was examined in therapeutic administration (posttreatment). Treatment with anti-Thy1.2 mAb significantly suppressed the therapeutic effect of ZSS (Table 4, VII). This suggests that ZSS might augment the activity of antitumor effector cells in therapeutic administration, and that T-cells take part in such antitumor activity. The present results suggest that ZSS might exhibit both direct cytotoxic activity and indirect host-mediated antitumor activity.

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