Cytotoxic T-Cell-mediated Antitumor Effect of Levamisole against Murine Syngeneic Fibrosarcoma

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ABSTRACT

Growth of the secondary Meth 1 tumors, which had been inoculated s.c. in the abdomens of BALB/c mice bearing primary Meth 1 tumors in the flanks, was inhibited as compared with that of tumors in the normal mice, suggesting the development of concomitant antitumor immunity. When levamisole (LMS, 0.625 or 2.5 mg/kg) was administered i.p. to the Meth 1-bearing mice daily before or after secondary inoculation, growth inhibition of secondary tumors was augmented. This effect of LMS was suggested to be tumor specific as evidenced by no growth inhibition of secondary Meth A tumors in mice bearing primary Meth 1 tumors.

The spleen cells of 11-day Meth 1-bearing mice exhibited growth-inhibitory activity against Meth 1 cells in the Winn assay. Administration of LMS augmented the growth-inhibitory activity. This effect of LMS was mediated by nonadherent spleen cells and completely lost by the preincubation with anti-Thy 1.2 antibody and complement. Cytotoxicity was detected in the spleen cells of Meth 1-bearing mice by 51Cr release assay after in vitro sensitization with mitomycin C-treated Meth 1 cells. In vivo administration of LMS augmented the cytotoxicity, which was tumor specific and completely lost by the preincubation of the spleen cells with anti-Thy 1.2 antibody and complement. These results suggest that the growth-inhibitory effect of LMS against secondary tumors was mediated by cytotoxic T-cells.

INTRODUCTION

Since Renoux and Renoux (19) reported the inhibitory effect of LMS against pulmonary metastases of murine syngeneic tumor (19), many findings have accumulated from experimental and clinical studies (2, 22). The antitumor effect of LMS against experimental tumors is supposed to be affected by the immune status of host, the dose of LMS, and the schedule of administration (2, 11, 19). In many experiments which showed the effectiveness of LMS as a single agent, tumors were highly immunogenic and slow growing (2). LMS was also reported to be more effective against metastases than against primary tumor (7, 17, 19, 23) and in combination with chemotherapeutic agents than by single administration (4, 14, 23).

There have been few reports in which the mechanism of the antitumor effect of LMS was investigated in relation to the antitumor immunity. It had been found that mice with progressively growing tumors might be resistant to reinoculation of the same tumor cells (5, 13). Such a phenomenon was called concomitant antitumor immunity and considered to be one of the evidences which suggested the existence of tumor-specific antigens in many experimental tumors. The present study was carried out in order to understand the effect of LMS against concomitant antitumor immunity and its effector mechanism in mice bearing syngeneic fibrosarcoma.

MATERIALS AND METHODS

Animals. Male BALB/c mice were obtained from Japan Charles River Co. (Kanagawa, Japan). Normal 9- to 10-week-old mice were used for experiments.

Tumors. Fibrosarcoma Meth 1 cells, induced in a male BALB/c mouse by methylcholanthrene in our laboratory, have been maintained in the ascites form in syngeneic BALB/c mice for several years. Sarcoma Meth A cells (16) have been maintained in the ascites form in syngeneic BALB/c mice. In vivo experiments, Meth 1 or Meth A cells were suspended in 0.1 ml of Dulbecco’s phosphate-buffered saline (Ca2+-, Mg2+-free) and inoculated s.c. in the flanks or abdomens of BALB/c mice. When 3 x 10^6 Meth 1 cells were inoculated s.c. in the flanks of BALB/c mice, all mice died from tumor progression and their survival time was between 30 and 60 days.

Antitumor Effect. After measuring the length and width of the tumor, tumor volume was calculated by the following formula according to the method of the National Cancer Institute (9):

\[
\text{Tumor volume (cu mm)} = \frac{\text{Length (mm)} \times \text{width (mm)}^2}{2}
\]

Drug. LMS (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) was dissolved in sterile 0.9% NaCl solution.

Culture Medium. Roswell Park Memorial Institute Tissue Culture Medium 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), 50 µg 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 100 units penicillin (Meiji Seika Co., Tokyo, Japan) per ml, and 100 µg streptomycin (Kyowa Hakko Kogyo Co.) per ml was used as culture medium.

Preparation of Spleen Cells. Spleens were collected aseptically from normal or Meth 1-bearing mice, pooled on ice, and squeezed with 2 glass slides in Hanks’ balanced salt solution (Grand Island Biological Co.) or in culture medium. The cell suspensions were passed through a layer of 4 sheets of gauze to remove residual large fragments and centrifuged at 100 x g for 5 min. Then cells were suspended in Hanks’ balanced salt solution or in culture medium. The cell suspensions were adjusted to the described cell concentration after counting viability by the trypan blue dye (Tokyo Kasei Co., Tokyo, Japan) exclusion method. Viability was over 80%.

Winn Assay. According to the method of Winn et al. (26), spleen cells and 3 x 10^6 Meth 1 cells were mixed in 0.2 ml of Hanks’ balanced salt solution and inoculated s.c. in the flanks of normal mice. Tumor volume was measured after 1 or 2 weeks, and antitumor activity of spleen cells was examined by the growth inhibition of tumor cells. The control group was inoculated with Meth 1 cells alone.

Induction of in Vitro Cytotoxicity. Spleen cells were stimulated in vitro with mitomycin C-treated Meth 1 cells prepared by the following method. Meth 1 cells (10^6) were incubated with 100 µg of mitomycin C (Kyowa Hakko Kogyo Co.) per ml at 37°C for 1 hr in Dulbecco’s...
phosphate-buffered saline (Ca^{2+}, Mg^{2+}-free). Then Meth 1 cells were washed 3 times with culture medium. Five × 10^6 spleen cells and mitomycin C-treated Meth 1 cells suspended in 2 ml of culture medium were added to Falcon 2058 tubes (Falcon Plastics, Oxnard, Calif.). Triplicate tubes were incubated for 5 days at 37° in a humidified atmosphere containing 5% CO_2 in air.

**In Vitro Cytotoxicity Test.** Cytotoxicity of spleen cells induced by in vitro stimulation with mitomycin C-treated Meth 1 cells was determined by the method of Brunner et al. (3) with a slight modification. Briefly, 10^6 Meth 1 or Meth A cells in 1 ml of culture medium were labeled with 200 μCi of 51Cr (Japan Atomic Energy Research Institute, Tokyo, Japan) at 37° for 1 hr and washed 3 times with culture medium. Effector cells (10^5) and labeled target cells (10^5) in 0.2 ml of culture medium in Linbro 76-023-05 v-bottom microplates (Linbro Scientific Inc., New Haven, Conn.) were centrifuged at 200 × g for 1 min and incubated at 37° for 5 hr in a humidified atmosphere containing 5% CO_2 in air. Then the microplates were centrifuged at 400 × g for 5 min, and the amount of 51Cr released in 0.1 ml of supernatant was measured with a well-type gamma counter (Aloka Co., Tokyo, Japan). The percentage of 51Cr release was calculated from counts of triplicate wells by the following formula:

\[
\text{% of cytotoxicity} = \frac{\text{Test release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100.
\]

Spontaneous release was the cpm released from the target cells incubated in the culture medium without effector cells. Maximum release was determined by the release from the target cells incubated in saponin solution (Toa Medical Electronics Co., Ltd., Hyogo, Japan). Spontaneous release was below 20%, and maximum release was about 80% of the cpm of the target cells (10^6).

**Treatment with Anti-Thy 1.2 Antibody and Complement.** Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer (pH 7.3; Grand Island Biological Co.) and 0.3% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used for preparation of spleen cells and for dilution of antibody or complement. An equal volume of 2 × 10^6 spleen cells, 1:5000 diluted anti-Thy 1.2 antibody (Olac 1976, Ltd., Bicester, England), and 1:10 diluted rabbit complement (Cederlane Laboratory Ltd., London, Canada) was mixed and incubated at 37° for 1 hr under gentle shaking. Spleen cells were washed 2 times with Hanks' balanced salt solution or culture medium and used in the Winn assay or 51Cr release assay, respectively.

**Separation of Adherent or Nonadherent Cells from Spleen Cells.** Five × 10^7 spleen cells suspended in 10 ml of culture medium were incubated in plastic dishes (No. 3003; Falcon Plastics) at 37° for 1 hr in a humidified atmosphere containing 5% CO_2 in air. After shaking the plastic dishes gently, nonadherent cells were collected. The plastic dishes were further washed twice with cold Dulbecco's phosphate-buffered saline (Ca^{2+}, Mg^{2+}-free), and adherent cells were collected with a rubber policeman. Nonadherent or adherent cells were centrifuged at 100 × g for 5 min, suspended in Hanks' balanced salt solution, and used in the Winn assay.

**Statistical Analysis.** Experimental results were analyzed for significance by Student's t test.

**RESULTS**

**Growth Inhibition of Secondary Tumors in Meth 1-bearing Mice.** The development of antitumor concomitant immunity was investigated in Meth 1-bearing mice. BALB/c mice, which were primarily inoculated s.c. with 3 × 10^6 Meth 1 cells in the flanks on Day 0, were secondarily inoculated s.c. with 3 × 10^6 Meth 1 cells in the abdomens, distant from the site of primary tumor, on Day 7, 14, or 21, and growth of secondary tumors was compared with that of tumors inoculated into normal mice (Table 1). V growth inhibited secondary tumors inoculated on Day 14 was inhibited more remarkably than that of those inoculated on Day 7. Significant growth inhibition was also detected in the secondary tumors inoculated on Day 21, when the primary tumors were in the advanced stage.

The relation between inoculum size and tumor growth was investigated in the secondary tumors inoculated on Day 11 (Table 2). When 3 × 10^6 Meth 1 cells were inoculated secondarily, their growth was significantly inhibited. Moderate growth inhibition was also demonstrated when 3 × 10^6 Meth 1 cells were inoculated secondarily. However, growth inhibition of secondary 3 × 10^7 Meth 1 cells was just slight.

**Growth-inhibitory Effect of LMS against Primary or Secondary Tumors.** Following the results of Tables 1 and 2, the inoculum size of 3 × 10^6 cells was adopted for a secondary tumor which was to evaluate the antitumor effect of LMS and was inoculated 11 days after the primary inoculation. BALB/c mice, which were primarily inoculated s.c. with 3 × 10^6 Meth 1 cells in the flanks on Day 0, were given LMS i.p. once a day for 10 days starting from Day 1, and the antitumor effect of LMS against primary or secondary tumors was examined (Table 3). LMS did not exhibit an antitumor effect against primary tumors at any dose from 0.156 to 10 mg/kg but inhibited the growth of secondary tumors. On Day 22, the secondary tumors of Meth 1-bearing mice administered LMS (0.625 or 2.5 mg/kg) were significantly smaller than those of Meth 1-bearing mice not administered LMS.

**Tumor Specificity of Growth-inhibitory Effect of LMS against Secondary Tumors.** To examine the tumor specificity of concomitant immunity induced in Meth 1-bearing mice, Meth 1 or Meth A cells were selected as secondary tumors and inoculated into Meth 1-bearing mice (Table 4). As already shown in Table 3, LMS augmented the growth inhibition of secondary Meth 1 tumors in Meth 1-bearing mice. On the other hand, when Meth A cells were inoculated into Meth 1-bearing mice, growth of Meth A cells was to the same degree as that of Meth A cells inoculated into normal mice. LMS did not affect the growth of Meth A cells in Meth 1-bearing mice. These results suggest that the growth-inhibitory effect of LMS against secondary tumors was significantly more effective when the inoculation size was smaller.
The effect of LMS against the growth of secondary tumors in Meth 1-bearing mice might be antigen specific.

**Schedule of Administration of LMS.** Influence of administration schedule of LMS on the growth of secondary tumors was examined (Table 5). LMS (2.5 mg/kg) administered not only before secondary inoculation, from Days 1 to 10, but also after secondary inoculation, from Days 11 to 20, inhibited the growth of secondary tumors on Day 22. On both schedules of administration, LMS did not affect the growth of primary tumors.

**Tumor Growth-inhibitory Activity of Spleen Cells in the Winn Assay.** To substantiate the development of concomitant immunity in Meth 1-bearing mice, spleen cells were tested for inhibition of growth of Meth 1 cells by the Winn assay (Table 6). With any effector:target ratio examined, normal spleen cells did not inhibit the growth of Meth 1 cells at all. On the other hand, spleen cells of Meth 1-bearing mice inhibited significantly the growth of Meth 1 cells with an effectortarget ratio of 50:1 or 200:1. These results indicate that **in vivo** resistance to secondary tumors in Meth 1-bearing mice was explicable by the antitumor activity detected at least in the spleen cells.

**Effect of LMS against Tumor Growth-inhibitory Activity of Spleen Cells.** Effect of LMS against tumor growth-inhibitory activity of spleen cells was shown in Table 7. Spleen cells of 11- or 16-day Meth 1-bearing mice given or not given LMS were used as tumor-bearing spleen cells, and the Winn assay was performed with an effector:target ratio of 50:1 or 200:1. These results indicate that **in vivo** resistance to secondary tumors in Meth 1-bearing mice was explicable by the antitumor activity detected at least in the spleen cells.

**Characterization of Effector Cells in the Spleen Cells of Meth 1-bearing Mice Stimulated with LMS.** The effector mechanism of antitumor immunity augmented by LMS was examined by the Winn assay (Table 8). Growth-inhibitory activity of spleen cells of 11-day Meth 1-bearing mice was mediated by a cell population not adherent to a plastic dish and was lost by preincubation with anti-Thy 1.2 antibody and complement.
Adherent cells did not affect the growth of Meth 1 cells. Similar results were obtained in the spleen cells of the Meth 1-bearing mice administered LMS (2.5 mg/kg). LMS augmented the growth-inhibitory activity of whole spleen cells or nonadherent cells, although not statistically significantly with the effector:target ratio of 50:1 used in this experiment. These results suggest that T-cells were essential to the growth-inhibitory activity of spleen cells of Meth 1-bearing mice and LMS augmented the growth-inhibitory activity through the stimulation of T-cell function.

**Augmentation of Antitumor Cytotoxicity by LMS.** An *in vitro* cytotoxicity test was performed by 51Cr release assay in order to measure the cytotoxic activity of spleen cells in Meth 1-bearing mice (Table 9). Spleen cells of Meth 1-bearing mice became cytotoxic by *in vitro* stimulation with mitomycin C-treated Meth 1 cells. Without *in vitro* stimulation, no cytotoxicity was induced. Normal spleen cells did not exhibit cytotoxicity at all. In vivo administration of LMS (2.5 mg/kg) augmented the cytotoxic activity of spleen cells of Meth 1-bearing mice, which was detected after *in vitro* stimulation.

**Characterization of Effector Cells Responsible for Antitumor Cytotoxicity.** Cytotoxicity of spleen cells stimulated *in vivo* with LMS and resensitized *in vitro* by mitomycin C-treated Meth 1 cells was completely lost by the treatment of spleen cells with anti-Thy 1.2 antibody and complement before the cytotoxic test (Table 10). Treatment with antibody or complement alone...
did not affect the cytotoxicity. The cytotoxicity was suggested to be specific to Meth 1 cells, because it was not demonstrated against Meth A cells.

**DISCUSSION**

In this paper, we reported the development of concomitant antitumor immunity in Meth 1-bearing mice and the augmentation of its immunity by LMS. The antitumor immunity induced in Meth 1-bearing mice was evidenced as follows. The growth of secondary Meth 1 tumors in Meth 1-bearing mice was inhibited significantly as compared with that of tumors in normal mice (Tables 1 and 2). Results of the Winn assay indicated that the spleen cells could mediate antitumor immunity (Table 6). When secondary inoculation was performed on Day 21 after primary inoculation (Table 1), the growth of secondary tumors was also inhibited significantly. Since the survival time of mice bearing the primary tumor alone was between 30 and 60 days as described in “Materials and Methods,” the strong antitumor immunity might be maintained in Meth 1-bearing mice even in the advanced stage of tumors. It was reported previously that suppressor immunocytes were induced in the early stage of tumor development, resulting in the suppression of antitumor immunity already on Day 7 after tumor inoculation (8). In BALB/c mice bearing Meth 1 cells, suppressor immunocytes might not be easily induced in the advanced stage of tumors.

**REFERENCES**


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