Therapeutic Effects of *Toxoplasma* Lysate Antigen on 20-Methylcholanthrene-Induced BALB/c Mouse Tumors

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ABSTRACT. Therapeutic effects of *Toxoplasma* lysate antigen (TLA) were studied in mice bearing the tumor in the second passage of 20-methylcholanthrene (MC)-induced tumor cells. Intramuscular administration of TLA 7 days after the tumor-cell inoculation caused apparent inhibition of the tumor growth on day 14. The second treatment facilitated the therapeutic effects. Intravenous transfer of spleen cells prepared from TLA-sensitized mice into tumor-bearing mice also represented the growth inhibitory effects. Prominent effects were seen when the transferred cells were prepared 5 days after sensitization of donor animals. The inhibitory effects were absent in the groups transferred only the adherent cells or the non-adherent cells prepared from sensitized mice. The strongest inhibitory effect was observed in the group to which both adherent and non-adherent spleen cells were transferred simultaneously from sensitized mice. In *in vitro* experiments, spleen cells obtained from sensitized mice showed cytolytic effect on P-815 or YAC-1 cells after the secondary stimulation *in vitro* with TLA. Large non-adherent cells containing densely packed granules were induced when cultured with the adherent cells obtained from sensitized mice. These results revealed that TLA can inhibit the growth of the chemically-induced transplantable tumors by activation of adherent and non-adherent spleen cells.—KEY WORDS: methylcholanthrene-induced tumor, mouse, spleen cell, therapeutic effect, *Toxoplasma* lysate antigen.

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It has recently been reported that antigen extracted from *Toxoplasma Gondii* (Tp) is able to activate human natural killer (NK) cells *in vitro* [12]. Mice treated with *Toxoplasma* lysate antigen (TLA) and infected with *Plasmodium berghei* or *Babesia rodhaini* are capable of surviving infections that would otherwise be fatal [4–6, 11, 13]. Interferon-gamma (IFN-gamma) and other lymphokines (LKs) are present in the serum of TLA-sensitized animals [3, 7, 14]. Incubation of TLA-sensitized mouse spleen cells with TLA *in vitro* causes induction of nonspecific cytotoxic cells that are capable of lysing target cells [2, 9]. The growth of allograftable Sarcoma-180 (S-180) tumors and isograftable Meth A tumors in BALB/c mice is also strongly inhibited by the administration of TLA [8]. These observations indicate that TLA causes a nonspecific stimulation of immunoprophylactic responses in mice. In this study, the therapeutic effect of TLA on mice with 20-methylcholanthrene (MC)-induced tumors was studied as a model for examining of the effects of TLA on spontaneous tumors.

MATERIALS AND METHODS

Experimental animals: Inbred male BALB/c mice were reared and maintained in this laboratory, and 4–6-week-old mice (10–20 g) were used in this experiment.

Preparation of Toxoplasma lysate antigen (TLA): TLA was prepared as described by Sakurai *et al.* [10] and Saito *et al.* [8]. For *in vivo* experiments, TLA was dissolved in 0.85% sodium chloride solution to make a final concentrations of 300, 1,000, or 2,000 μg/m*l* protein. The solutions were filter-sterilized with disposable syringe filter units (0.45 μm, Dismic-25, Toyo Roshi Co., Ltd., Japan) and stored at −20°C until use. For *in vitro* incubations, TLA was dissolved in RPMI-1640 (Flow Laboratories, U.K.) containing 12 mM HEPES, 100 units/m*l* potassium penicillin G, 100 μg/m*l* streptomycin sulfate, 0.1 μM sodium pyruvate, and 50 μM 2-mercaptoethanol to make a final concentration of 1,000 μg/m*l* protein. The solution was filter-sterilized as described above and stored at −20°C until use.

Preparation of methylcholanthrene-induced tumors: A paraffin pellet containing 0.2 mg of MC (Wako Pure Chemi. Ind., Tokyo, Japan) was inserted subcutaneously into the back of mouse. Tumors induced at the site of the implants approximately 3 months later were removed and divided into pieces of 10 mm^2 each. These were grafted

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subcutaneously on the backs of additional mice 8–12 weeks of age. Tumors from these animals were excised, chopped with scissors on ice, triturated between a pair of glass slides, and suspended in Dispase (Dispase grade II, Boehringer Mannheim Yamanouchi Co., Ltd., Tokyo, Japan). After stirring for 30 min at 37°C to digest connective tissue, the cell suspension was filtered twice through #40 stainless steel mesh and passed through a glass fiber column to eliminate large pieces. The cells were washed 3 times with HBSS (250×g, 6 min, 4°C) and suspended in RPMI-1640 to prepare a final concentration of 4×10^6 or 5×10^6 viable cells/m*l*. The cell viability was determined by the dye exclusion test using trypan blue. Additional mice were inoculated with 0.025 m*l* (1×10^5 cells/mouse) or 0.1 m*l* (5×10^5 cells/mouse) of these cell suspension.

Measurement of tumor growth: Nodular tumors formed by subcutaneous inoculation of MC tumor cells were measured with a pair of calipers. The area of each tumor was calculated as the product of the major and minor axes. Mean values from each group were compared with a Student's *t*-test.

Preparation of spleen cells: Spleens were excised from mice immediately after sacrifice, chopped with scissors, triturated between two glass slides, and suspended in HBSS. The spleen cells were passed through a #40 stainless steel mesh to eliminate large pieces of tissue. Red blood cells were hemolyzed with 0.83% ammonium chloride solution at 37°C. The cell suspension was filtered through a glass fiber column to obtain the lymphocyte fraction according to the method of Conray-Ficoll [15]. Lymphoid cells were washed twice with HBSS and once with RPMI-1640 and suspended in RPMI-1640 before transfer to other animals.

For separation of spleen cell fraction, cell suspensions prepared as described above were resuspended in RPMI-1640 containing 10% heat inactivated fetal calf serum (FCS; Flow Laboratories, Australia) (10% FCS-RPMI) to make a final cell density of 3.0×10^6 cells/m*l*. The suspension was incubated in a culture flask for 12 hr. The culture flask was washed gently with HBSS to collect non-adherent (NA) cells. Adherent (A) cells were removed from the flask with a rubber policeman. Both fractions were washed once with HBSS before transfer to mice.

Preparation of effector cells: Spleen cells prepared from TLA-sensitized or non-sensitized mice were suspended to make a density of 3×10^6 cells/m*l* in 10% FCS-RPMI containing with or without 30 μg/m*l* TLA and incubated for 6 days. The cells were then washed twice with RPMI-1640, resuspended to the same density, and used as effector cells.

Cytotoxicity test: The mouse mast cell sarcoma P-815 and the mouse T cell lymphoma YAC-1 were kindly supplied by Dr. Tsuneo Kamiyama, Department of Animal Epidemiology, National Institute of Health, Japan, and used as target cell which is resistant or sensitive to natural killer cells.

Cell suspensions prepared at densities of 1×10^7 viable cells/m*l* were incubated with 5.55 MBq of ^{51}Cr (Na_2CrO_4, specific activity of 1.5 GBq/m*l*, Japan Atomic Energy Research Institute, Tokyo, Japan) with occasional agitation for 60 min. The ^{51}Cr-labeled cells were washed with phosphate buffered physiological saline (PBS) and then with RPMI-1640 [1]. The pellet was resuspended in 10% FCS-RPMI to make a final density of 1×10^5 cells/m*l*.

Culture tubes were filled with 150 μl of the ^{51}Cr-labeled target cell suspension (1.5×10^4 cells) and 150 μl of the effector cell suspension (3.0×10^2 cells) to adjust the ratio of effector: target cells (E:T ratio) to 50:1. One m*l* of 10% FCS-RPMI was added to each tube and incubated for 20 hr. Each tube was centrifuged (1,000×g, 5 min, room temperature) after incubation and 1.2 m*l* of the supernatant and 0.1 m*l* of the pellet were sampled for counting in a gamma-scintillation counter (Packard auto-gamma 5650; United Technologies Packard, U.S.A.). The release of ^{51}Cr from each sample was measured in triplicate. Non-specific release of ^{51}Cr was measured in samples to which 150 μl of 10% FCS-RPMI was added in place of the effector cells.

Cytotoxicity of effector cells was calculated according to the following formula:

$$\text{Cytotoxicity (\%)} = 100 \times \{\text{Release of sample } ^{51}Cr \text{ (cpm)} - \text{release of nonspecific } ^{51}Cr \text{ (cpm)}\} / \{\text{Release of total } ^{51}Cr \text{ (cpm)} - \text{Release of nonspecific } ^{51}Cr \text{ (cpm)}\}$$

Analysis of mouse spleen cells by automatic cell sorting: Cultured mouse spleen cells were washed once with PBS and suspended in 1 m*l* of 10 mM HEPES buffer containing 0.1% BSA. The suspension was filtered through a 40 μm nylon filter and analyzed with an automatic cell sorter CS-20, Argon ion laser (400 mw, 488 nm, Showa Denko Co., Tokyo, Japan). Approximately 2,000 cells were processed in each group.

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RESULTS

Therapeutic effects of TLA: As shown in Fig. 1, each group of 5 mice was administered intramuscularly with either 0.1 m*l* of physiological saline (control) or 0.1 m*l* of physiological saline containing 30 (T30), 100 (T100), and 200 (T200) μg TLA weekly starting on day 7.

Mean tumor size 14 days after the inoculation was 48 mm^2 in control group and 25 mm^2, 22 mm^2 and 28 mm^2 in T30, T100, and T200, respectively. Groups receiving TLA had significantly smaller tumors than control group. On day 21, the differences among the control and TLA treated groups were highly significant ($p<0.01$). Mean tumor size of T100 was smallest whereas no statistical differences were observed among the TLA treated groups.

Effect of TLA-sensitized spleen cells on tumor growth: As shown in Fig. 2, 21 mice were divided into 5 groups on day 7 after inoculation of 5×10^5 tumor cells per mouse. Each animal received 2×10^6 spleen cells (containing both adherent and non-adherent cells) from TLA-sensitized or non-sensitized mice via the caudal vein. Cells prepared from the mice on day 1, 3, 5 and 7 after intramuscular administration of TLA were transferred into the tumor-bearing mice of 4 groups, 1DT, 3DT, 5DT, and 7DT, respectively. Transfer of spleen cells was carried out on day 7 and day 14 after tumor transplantation. Changes in tumor size were measured every 2 days starting on day 8. Mean tumor size on day 21, was 512 mm^2 in control group, 396 mm^2 in 1DT, 373 mm^2 in 3DT, 302 mm^2 in 5DT, and 405 mm^2 in 7DT. Tumor growth was inhibited significantly in 1DT and 3DT ($p<0.05$) and in 5DT ($p<0.01$), but not in 7DT.

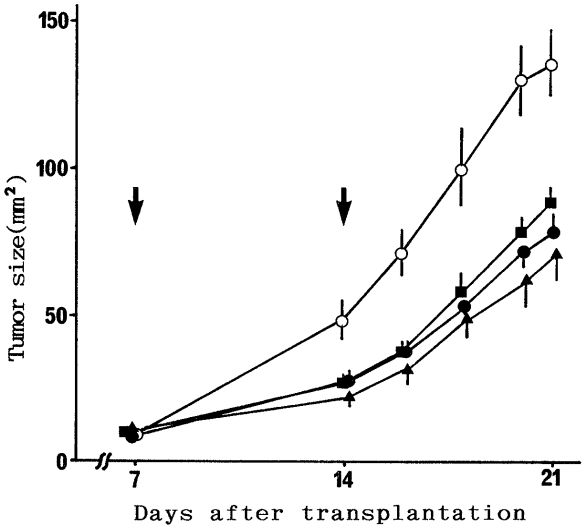

Fig. 1. Growth inhibitory effects of TLA on the Methylcholanthrene-induced tumors (2nd passage). (○), Control; (●), TLA 30 μg/mouse; (▲), TLA 100 μg/mouse; (■), TLA 200 μg/mouse. Each point and vertical bar represent the mean ±SE. Arrows indicate the day of TLA administration.

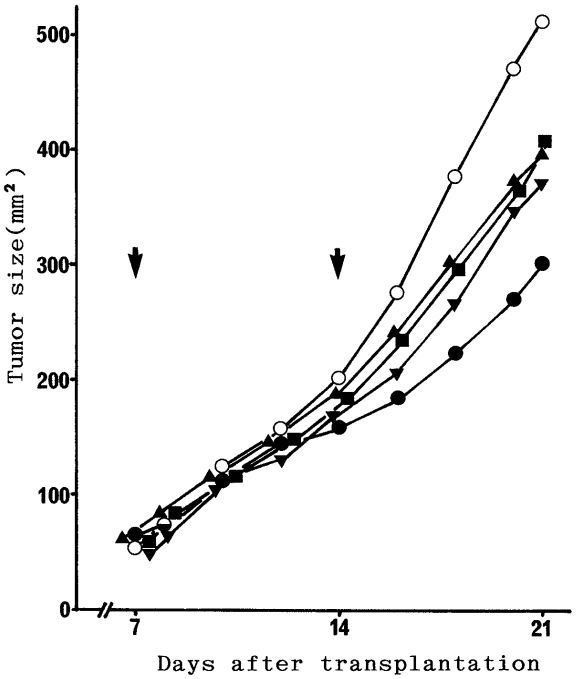

Fig. 2. Tumor growth inhibition by transferring the spleen cells prepared from TLA-sensitized mice. Spleen cells were prepared on day 1 (▲), day 3 (▼), day 5 (●), or day 7 (■) after intramuscular administration of TLA, and the control group (○) received unsensitized cells. Arrows indicate the day when the spleen cells were transferred.

In order to clarify the population of spleen cells that might play a role in tumor inhibition, tumor-bearing mice received the adherent (A) cells and/or non-adherent (NA) cells prepared from the mice 14 days after TLA-sensitization or from the non-sensitized mice (Fig. 3). Proportions of Thy-1, Ig, or asialo GM1 positive cells in NA cells prepared from non-sensitized mice (NAC) and from TLA-sensitized mice (NAT) were 50.4, 23.3 or 4.5%, and 50.2, 25.4 or 4.7%, respectively. Proportions of macrophage in adherent cells from the former (AC) and the latter (AT) animals were 68.0 and 66.0%, respectively. The animals of each group received 2×10^6 NA cells and/or 2×10^5 A cells at weekly intervals for five consecutive weeks.

Mean tumor size 33 days after tumor inoculation was 180.6 mm^2 in control group, 156.6 mm^2 in Group NAC, 133.4 mm^2 in Group AC, 189.6 mm^2

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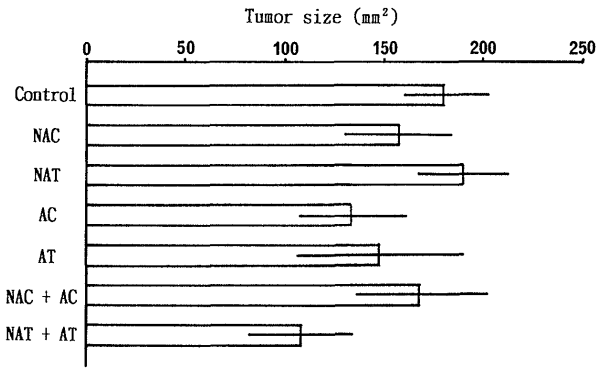

Fig. 3. Tumor growth in mice receiving adherent (A) and/or non-adherent (NA) spleen cells from either TLA-sensitized mice (T), or non-sensitized mice (C), respectively. The data shown are the mean tumor size±SE of five mice 33 days after inoculation.

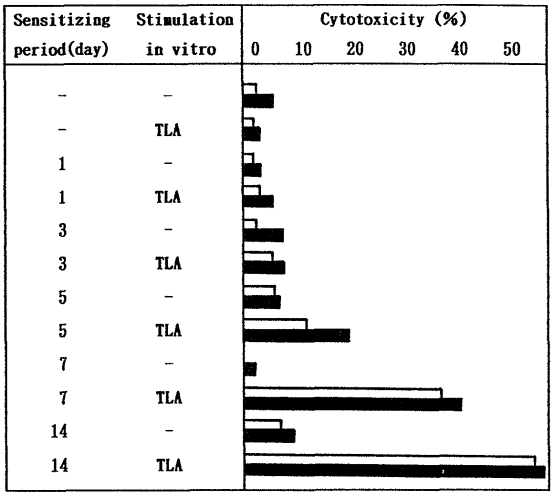

Fig. 4. Effect of cytotoxicity in terms of TLA-sensitizing procedure. Spleen cells prepared from the mice sensitized with TLA 1, 3, 5, 7, and 14 days before sacrifice were restimulated *in vitro* with TLA. Cytotoxic activities against P-815 (□) and YAC-1 (■) were measured 6 days after secondary stimulation.

in Group NAT, 148.5 mm^2 in Group AT, 169.0 mm^2 in Group NAC + AC, and 108.3 mm^2 in Group NAT + AT. Inhibition of tumor growth in Group NAT + AT was the most prominent among other Groups.

Relationship between cytotoxicity and sensitizing period: Spleen cells were prepared from TLA-sensitized mice on 1, 3, 5, 7, and 14 day and restimulated with TLA *in vitro*. The cytotoxicity test was carried out 6 days after the incubation with target cells. Spleen cells prepared within 3 days after sensitization showed little cytotoxicity to P-815 or YAC-1 target cells (Fig. 4). However, secondary stimulation with TLA *in vitro* induced marked cytotoxicity 5 days after sensitization *in vivo*, especially in the groups of 7 and 14 days-sensitizing periods. These values were significantly different from those in the same groups without secondary stimulation ($p<0.001$).

Morphological analysis of nucleated cells: The cell size and the quantity of intracellular granules of NA cells were measured in the four cases where NA and A cells prepared from TLA-sensitized or non-sensitized mice were cultured concurrently, i.e. NAC + AC, NAC + AT, NAT + AC, and NAT + AT (Fig. 5). After a 6-day incubation, NA cells in each group were collected and analysed with a cell sorter. The proportion of cells larger than the 40th channel on the abscissa (ca. 21.6 μm) was calculated for each group. Proportions were 61.0% for Group NAC + AC, 68.4% for Group NAC + AT, 65.2% for Group NAT + AC, and 77.8% for Group NAT + AT. Large mononuclear cells containing densely-packed intracellular granules were present in Group NAT + AT.

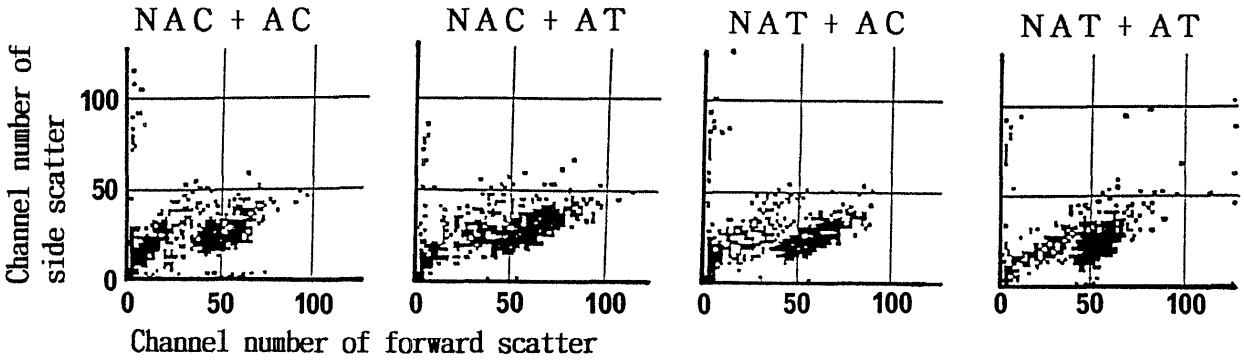

Fig. 5. Cell-sorter profiles of non-adherent spleen cells cultured with adherent cells in various combinations indicated in the figures (Abbreviations mean the same groups as in Fig. 3). The axis of abscissas and ordinate show the cell size and the quantity of intracellular granules, respectively.

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DISCUSSION

The therapeutic effects of TLA on MC-induced tumor cells were studied as a model for evaluating the clinical availability of this compound for spontaneous tumors. Weekly intramuscular doses of TLA to mice with MC-induced tumors inhibited tumor growth in the cases treated 7 days or later after tumor transplantation. Previous studies have reported that TLA inhibits the *in vivo* growth of established allograftable Sarcoma 180 tumor cells and established isograftable Meth A tumor cells [8]. Results in this study using second passaged tumor suggest that TLA can be applied in veterinary medicine for the control of spontaneous tumors.

Significant inhibition of tumor growth occurred in our study when TLA was administered at doses of 30, 100, and 200 μg/mouse. While inhibition of tumor growth was most prominent at doses of 100 μg/mouse, there were no significant differences among any of the three regimens. Since cytotoxicity of lymphoid cells collected from the spleens of TLA-sensitized mice was larger in mice that received 100 μg than 30 μg of TLA at biweekly intervals [9], we consider a single 100 μg dose of TLA to be sufficient for stimulating biological effects.

The growth of tumors was inhibited within 7 days by administration of a single dose of TLA at 100 μg/mouse and by transfer of spleen lymphoid cells from mice that had been sensitized with 100 μg of TLA 5 days earlier. Spleen cells from unsensitized mice and mice that had been sensitized with 2 doses of TLA at biweekly intervals were collected and separated into A and NA cell populations in order to determine the underlying mechanisms of TLA activity. Adherent and NA cells were transferred to mice with MC-induced tumors in a number of different combinations. Tumor growth was inhibited evidently in mice that received a mixture of A and NA cells from TLA-sensitized mice. Co-incubation of these two cell populations and subsequent analysis by cell sorter revealed the induction of large NA cells that contained large numbers of intracellular granules. An increase in the cytotoxic activity of spleen cells harvested between 5 and 14 days after sensitization with TLA and restimulation with TLA *in vitro* indicated that cytotoxic cells, such as NK sensitive and/or resistant killer cells, also participate in the anti-tumor activity of TLA. Anti-tumor immunity is said to involve the response of immunologically competent cells after induction by antigen-presenting cells. These results, in consequence, revealed that TLA can inhibit the growth of the chemically induced transplantable tumor on the basis of activation of A and NA cells, especially in the spleen.

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