

## Antitumor Activity of *Toxoplasma* Lysate Antigen against Methylcholanthrene-Induced Tumor-Bearing Rats

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**ABSTRACT.** Growth of the tumor autoinduced by 20-methylcholanthrene (MC) in rats was inhibited after administration of *Toxoplasma* lysate antigen (TLA). The antitumor activity of TLA was most obvious in the early stage of tumoral growth. When TLA was administered to rats before the appearance of tumor, tumor formation was delayed slightly. Histopathological studies revealed dense growths of spindle tumor cells in untreated control rat, while enlarged central necrosis with the infiltration of lymphocytes and neutrophils was apparent in TLA-treated rats. According to the immunohistological examination of tumor tissue with anti-Thy-1 antibody, the rats treated with TLA showed large Thy-1 positive granular cells, whereas the untreated rats indicated only a few small Thy-1 positive cells. These observations indicate that TLA is a useful modifier of biological responses to MC-induced tumors.—**KEY WORDS:** antitumor activity, methylcholanthrene-autoinduced tumor, rat, Thy-1 positive granular cell, *Toxoplasma* lysate antigen.

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Mice treated with *Toxoplasma* lysate antigen (TLA) was protected preferentially from the infection with *Plasmodium berghei* or *Babesia rodhaini*, and survived the challenge fatal to non-treated animals [7, 8, 14, 16]. Interferon-gamma (IFN-gamma) and other lymphokines (LKs) were present in the serum of these animals [5, 7, 9, 17]. Furthermore, cytotoxic cells were induced when spleen cells harvested from TLA-sensitized mice were incubated with TLA *in vitro* [4, 6, 12]. Sharma *et al.* [15] have also reported that TLA activated human natural killer (NK) cells *in vitro*. In the light of these observations, it can be presumed that TLA might cause nonspecific activation of immunoproliferative mechanisms.

Further studies on the antitumor activity of TLA revealed that the intramuscular administration of TLA strongly inhibited the growth of allogenic or isogenic tumors, i.e. Sarcoma-180 (S-180) or Meth A [11]. Moreover, an apparent antitumor effect of TLA was observed even in the mice bearing 20-methylcholanthrene (MC)-induced tumor [6].

In this report, rats with the MC-autoinduced tumors were examined as a therapeutic model for the TLA treatment.

### MATERIALS AND METHODS

**Rats:** A total of 39 male rats of the Wistar-

Imamichi strain of 6 to 8 weeks of age were used for the experiments except for the hematological study, in which 3 mature female rats of the Rowett strain were used. All animals were raised and maintained in our laboratory.

**Preparation of *Toxoplasma* lysate antigen (TLA):** TLA was prepared according to the method described previously [3, 13].

In brief, after the centrifugation of crude antigen solutions at  $144,000 \times g$  for 120 min, the supernatant was used as TLA preparation throughout the experiment.

**Induction of tumors with 20-methylcholanthrene (MC):** A solution of MC (Wako Pure Chemi. Ind., Tokyo) in paraffin at a final concentration of 5 mg/ml was divided into 0.1 ml aliquots, and allowed to cool. A pellet was implanted subcutaneously on the back of rat, which was kept under observation until the tumoral growth (for about 4 or 5 months).

**Hematology:** Blood sample was collected by heart puncture from the rat with or without TLA treatment. Red blood cell counts, white blood cell counts, hematocrit (Ht; capillary method), total serum protein (STP; refractometry), glutamic-oxaloacetic transaminase activity (SGOT; karmen's method), glutamic-pyruvic transaminase (SGPT; Karmen's method), lactate dehydrogenase (LDH; Wroblewski-Radu method), alkalinephosphatase (AIP; p-nitrophenol test), total bilirubin (Malloy-

Evelyn test), creatinine (alkaline picric acid test), and uric acid (urate oxidase test), were measured routinely.

*Measurement of tumor growth:* The major and minor axes of each nodule were measured using a pair of calipers and the tumor area calculated as the product of both axes. Mean values in each group were compared with a Student's *t*-test.

*Calculation of mononuclear cell distribution:* Spleen or liver was ground and the mononuclear cells were collected by the method of Conray 400-Ficoll [18]. After the number of nucleated cells in each organ was counted, the smears were prepared for the determination of lymphocyte subclasses.

*Calculation of lymphocyte subclasses:* Rabbit anti-rat Thy-1 serum (ATS) was prepared according to the method of Golub [2]. Confirmation of the specificity of this serum for T cells was carried out with the cytotoxicity tests according to the method described by Barker *et al.* [1]. Smears of mononuclear cells prepared from spleen or liver were incubated with ATS or fluorescein-isothiocyanate conjugated goat anti-rabbit IgG (Cappel Inc., U.S.A.). The ratio of ATS positive cells to the total mononuclear cell count was calculated. The ratio of IgG positive lymphocytes was determined in a similar manner by incubating the preparations with fluorescein-isothiocyanate conjugated anti-mouse IgG (Nippon Kotai Kenkyusho, Japan).

*Histopathology and immunohistochemistry:* Subcutaneous tumors were excised from rats and divided in half with a sharp scalpel. One piece of tissue was routinely processed for HE-staining and the other half was processed for immunohistochemical studies as described by Saint-Marie [10]. Fluorescein-isothiocyanate (FITC) conjugated mouse IgG antibodies (Sera-Lab Ltd., U. K.) against rat Thy-1, rat T helper cells and rat T cells (non-helper subset) were used.

## RESULTS

*Effects of TLA on the growth of MC-induced tumor:* Twenty rats with MC-induced tumors were divided into four groups of the same number according to the criteria listed in Fig. 1. Treatment with TLA was started when the tumor size reached to the diameter specified for the each group, i.e. about 3 mm, 10 mm or 18 mm. TLA dissolved in physiological saline was injected at a dose of 500

$\mu\text{g}/\text{rat}$  into the femoral muscle once a week for 5 weeks.

Effects of TLA depended on the tumor size at the beginning of treatment (Fig. 1). In the group "b" with tumors less than 10 mm in diameter, prominent inhibition of growth was observed in 2 of 5 animals, and growth delay occurred in the remaining 3 cases. In the group "c" with tumors about 10 mm in diameter, tumor growth was inhibited in 1 animal and only partially suppressed in 2 others. TLA had no effect on tumor growth in the group "d" with tumors larger than 10 mm in diameter.

In the second experiment, 5 rats bearing tumors about 10 mm in diameter were treated with TLA at a dose of 100  $\mu\text{g}/\text{rat}$  once a week for 8 weeks. Other 4 rats bearing the tumor of the same size were given intramuscularly physiological saline as a control group.

As shown in Fig. 2, the mean tumor area in the control group was 110  $\text{mm}^2$  at the beginning of the experiment and increased markedly to 421  $\text{mm}^2$  after 6 weeks and to 948  $\text{mm}^2$  after 9 weeks. Tumor growth was inhibited significantly ( $p < 0.05$ ) in the TLA-treated group, i.e. the initial tumor size (115  $\text{mm}^2$ ) was followed by slight increase to 191  $\text{mm}^2$  after 6 weeks and to 311  $\text{mm}^2$  after 9 weeks.

*Effect of TLA on the tumor induction by MC:* Ten male Wistar rats subcutaneously implanted of MC-containing paraffin pellets were divided into two equal groups. Animals in one group were not treated while those in the other group were administered 500  $\mu\text{g}$  of TLA once a month immediately after the implantation of the pellet. Tumor nodule was observed first in the untreated control group 124 days after the implantation (Fig. 3). Within the following 21 days, tumors were induced in all other untreated rats. TLA treatment slightly delayed the tumoral growth and one animal remained tumor-free throughout the experimental period.

*In vivo reactions to administration of TLA:* For the hematological, biochemical, immunological, and histopathological studies, MC-containing pellets were implanted into the femoral muscles of three Rowett rats. One animal was used as an untreated control (No. I), and other two rats (No. II, III) were treated with TLA according to the schedule indicated in Fig. 4. Hematological characteristics are listed in Table 1 where differences of the value in WBC, SGOT, LDH and uric acid were distinctive among the three rats. SGOT of the control rat was about three times as high as those of TLA treated

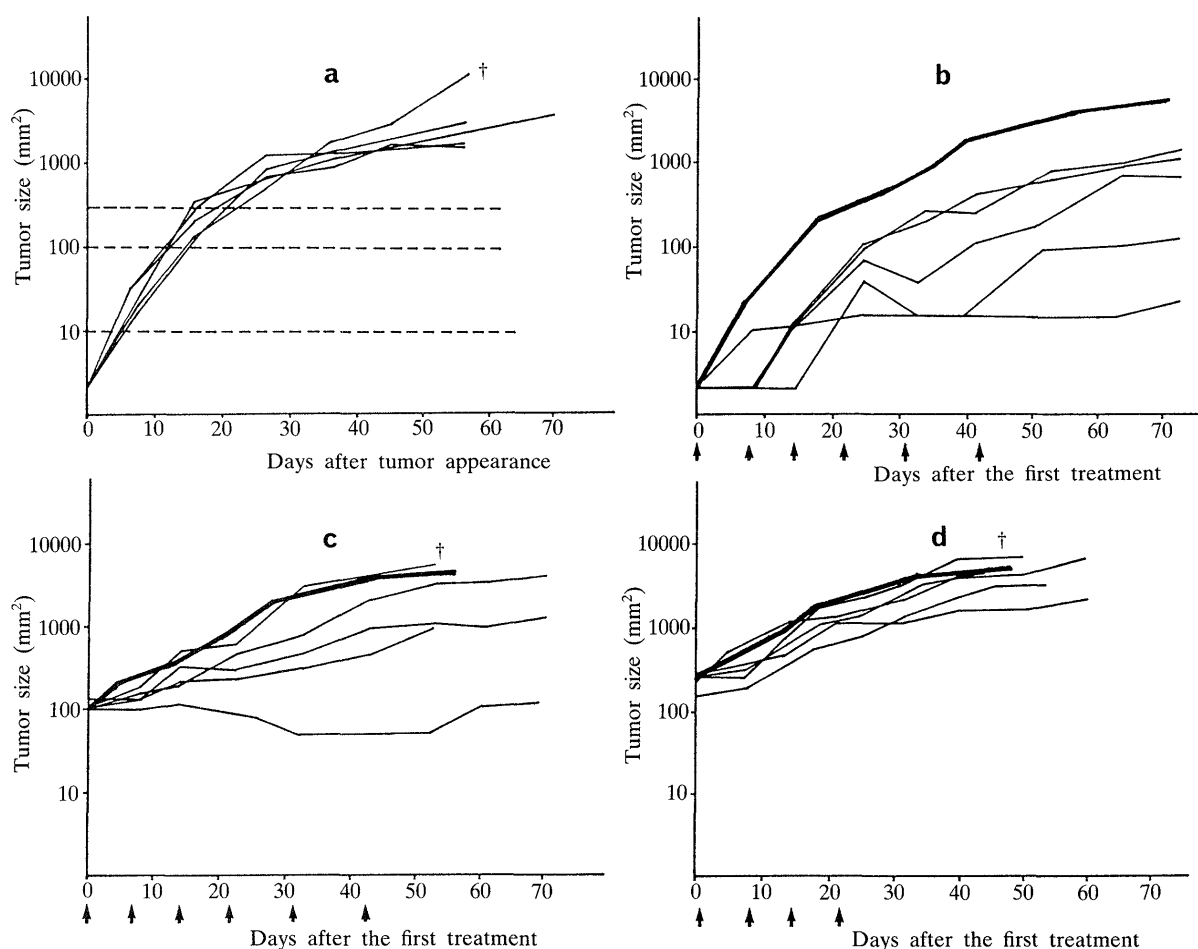


Fig. 1. Effects of TLA on the growth of MC-induced tumors at the various stages of growth. Group "a" was untreated control and groups "b", "c", or "d" were treated with TLA when the tumor diameter reached about 3, 10, or 18 mm, respectively. Each thick line in "b", "c" and "d" represents the average growth of control. Each dotted line in "a" represents tumor size when the treatment was started in the group "b", "c", or "d". Arrows indicate the day of TLA (500  $\mu$ g/rat) administration.

rats, No. II and No. III. LDH of the rat treated with TLA after tumor induction (No. II) was about one third that of control one, whereas LDH of the rat treated immediately after MC-pellet implantation (No. III) was about two times as high as that of control one.

Total lymphocytes counts of the spleen and liver were highest in No. II and lowest in No. III (Fig. 5). There were no marked differences among these rats concerning in the proportion of Ig positive cells, ATS positive cells and others.

Histopathological section of tumor tissue from No. I represented the characteristics of fibrosarcoma in which small necrotic foci were observed (Fig. 6). Tumor tissue of No. II, by contrast, showed honeycomb appearance with large necrotic foci, and intensive infiltration of lymphocytes and neutrophils was observed in the boundary of necrotic area (Fig.

7). Tumor of No. III was smaller and the central necrosis was more prominent than that of No. II (Fig. 8). Severe cell infiltration was also observed around the residual tumor tissue in the margin of the mass. Degenerative signs of tumor cells such as pyknosis and vacuolization were apparent in No. II and No. III.

Immunohistochemical examination revealed the existence of a few small Thy-1 positive cells in tumor tissue of No. I (Fig. 9a). In case of No. II, large granular Thy-1 positive cells were detected sporadically (Fig. 9b). Although no reactor was observed to the antibody against rat T helper cells, occasional labeling was evident with the antibody for non-helper subset. Immunohistochemical findings in tumor tissue from No. III were similar to those of No. II, where large granular Thy-1 positive cells were present in sporadic numbers (Fig. 9c).

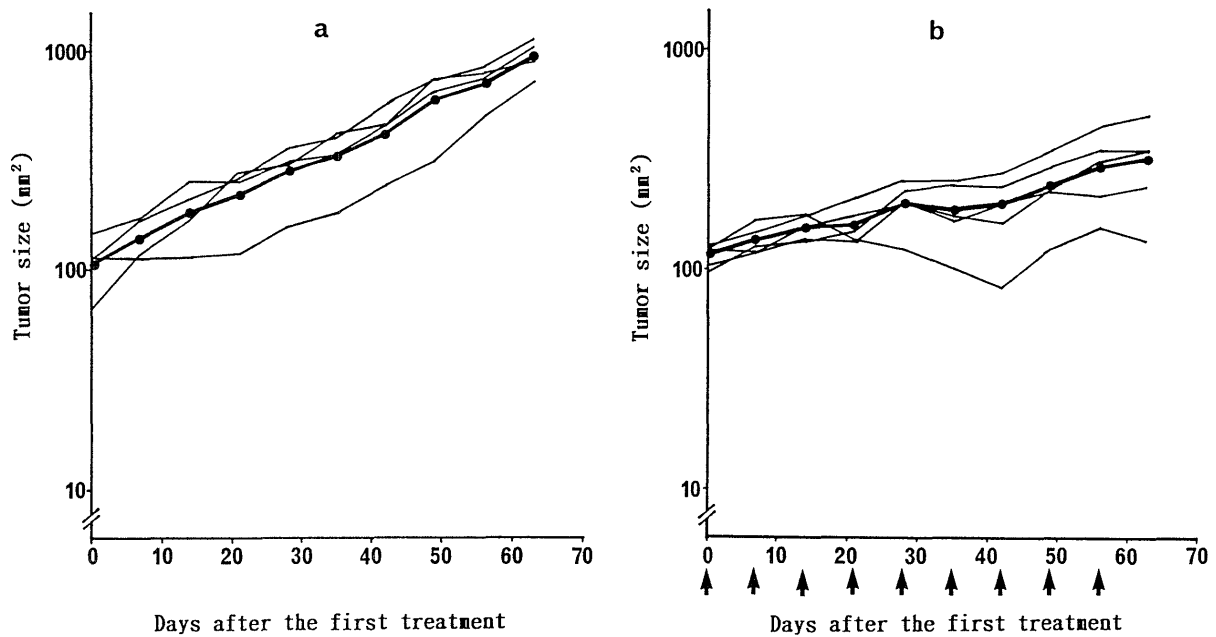


Fig. 2. Effects of the lower dose of TLA on the growth of MC-induced tumor. The experimental group "b" was intramuscularly administered 100  $\mu\text{g}$  of TLA once a week and the control group "a" was treated with saline as a same manner. Each thick line (●—●) represents the mean size and the arrows indicate the day of TLA administration.

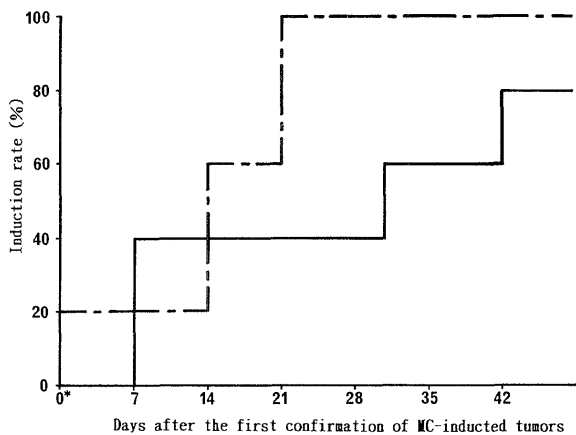


Fig. 3. Effects of TLA on the induction of tumor by MC. Two groups of 5 rats were implanted with a MC-treated paraffin pellet. One group was untreated (---). The other was given TLA i. m. at a dose of 500  $\mu\text{g}/\text{rat}$  at monthly intervals for 4 months (—). 0\*: 124 days after insertion of paraffin pellets.

Histological section of No. I liver showed focal necrosis where severe degenerative changes such as cloudy swelling and pyknosis were prominent (Fig. 10). The livers of TLA-treated rats (No. II and No. III) seemed more vivid than those of control animals while moderate degenerative changes of liver cells were still observed (Figs. 11 and 12). Intensive interstitial infiltrations composed of many neut-

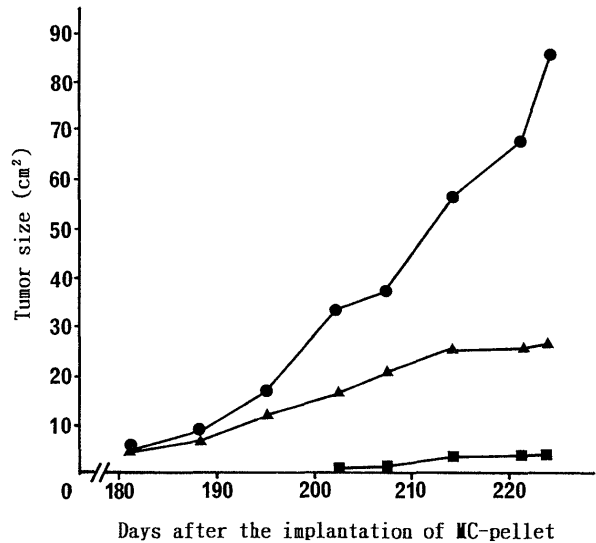


Fig. 4. Tumor growth of the rat implanted with MC-pellet for the further oncological studies. (●—●): Untreated rat (No. I). (▲—▲): The rat treated with TLA (500  $\mu\text{g}/\text{rat}$ ) at 2-week intervals for 6 weeks. Treatment began after the tumoral growth was observed (No. II). (■—■): The rat treated with TLA (500  $\mu\text{g}/\text{rat}$ ) weekly for 5 weeks. Treatment began immediately after the MC-containing paraffin pellet was implanted (No. III).

Table 1. Hematological characteristics of the tumor-bearing rat with or without TLA treatment

Case number <sup>a)</sup>	No. I	No. II	No. III
RBC ( $\times 10^4/\text{mm}^3$ )	312	328	387
WBC ( $\text{mm}^3$ )	6,700	11,500	4,100
Ht (%)	27	17	20
STP (g/dl)	5.2	3.6	5.3
SGOT (Karmen Unit)	304.2	88.6	98.5
SGPT (Karmen Unit)	24.0	27.4	23.7
LDH (Wroblewski Unit)	1,058	294	1,813
AlP (King-Armstrong Unit)	10.7	17.7	10.7
T-bilirubin (mg/dl)	0.33	0.19	0.14
Creatinine (mg/dl)	1.46	0.99	1.13
Uric acid (mg/dl)	3.99	3.73	11.56

a) Case numbers were identical to those in Fig. 4.

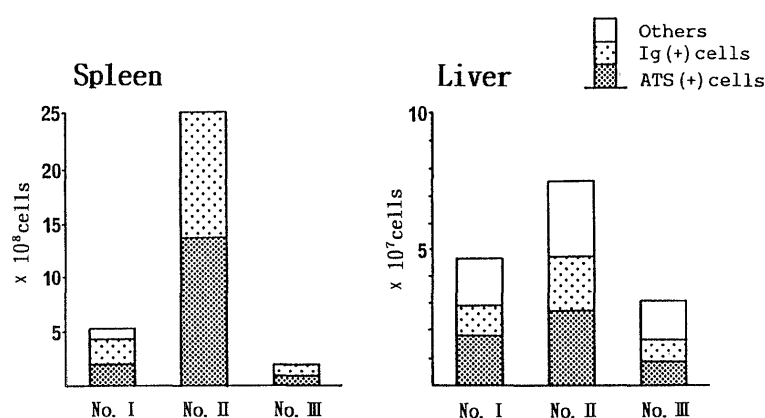


Fig. 5. Distribution ratio of mononuclear cell population in the spleen and liver of the MC-induced tumor bearing rat with or without TLA treatment. Case numbers (I-III) were identical to those in Fig. 4.

rophils, a few lymphocytes and large mononuclear cells were observed in cases of No. II and No. III. Furthermore, swelling of arteriolar endothelial cells was observed in No. III (Fig. 12).

#### DISCUSSION

Results of this study demonstrate that TLA inhibits the growth of MC-induced murine tumors. The inhibitory effects were most prominent in the case with the tumor smaller than 100 mm<sup>2</sup>. Weekly intramuscular administration of TLA at dosages of 100  $\mu\text{g}/\text{rat}$  was sufficient to cause these effects. Furthermore, slight inhibitory effects on the induction of tumor were evident when TLA was administered at the time of tumor induction.

Lymphocyte composition of the spleens and livers of these animals was examined to determine the mechanisms for the antitumor activity of TLA. When TLA was administered after the formation of

tumors, the number of splenic and hepatic lymphocytes was larger in TLA-treated animals than in untreated controls or rats dosed with TLA before formation of tumors. We reported significant increases in the numbers of B and T lymphocytes in the spleens and livers of TLA-treated mice within 10 days after infection with *Babesia* [4]. Similar responses occurred in the present study, although the number of animals in each experimental group was very limited.

Serum concentrations of SGOT and LDH were high in untreated rats, which corresponded to the histological observations of the liver. On the other hand, normal level of SGOT and moderate injury of liver cells were observed in the TLA-treated rats. These findings suggest the therapeutic effects of TLA concomitant of tumor inhibitory effect. Elevation of LDH is considered to result from the muscular disorders, and the rat treated with TLA just after MC-pellet implantation showed unexplain-

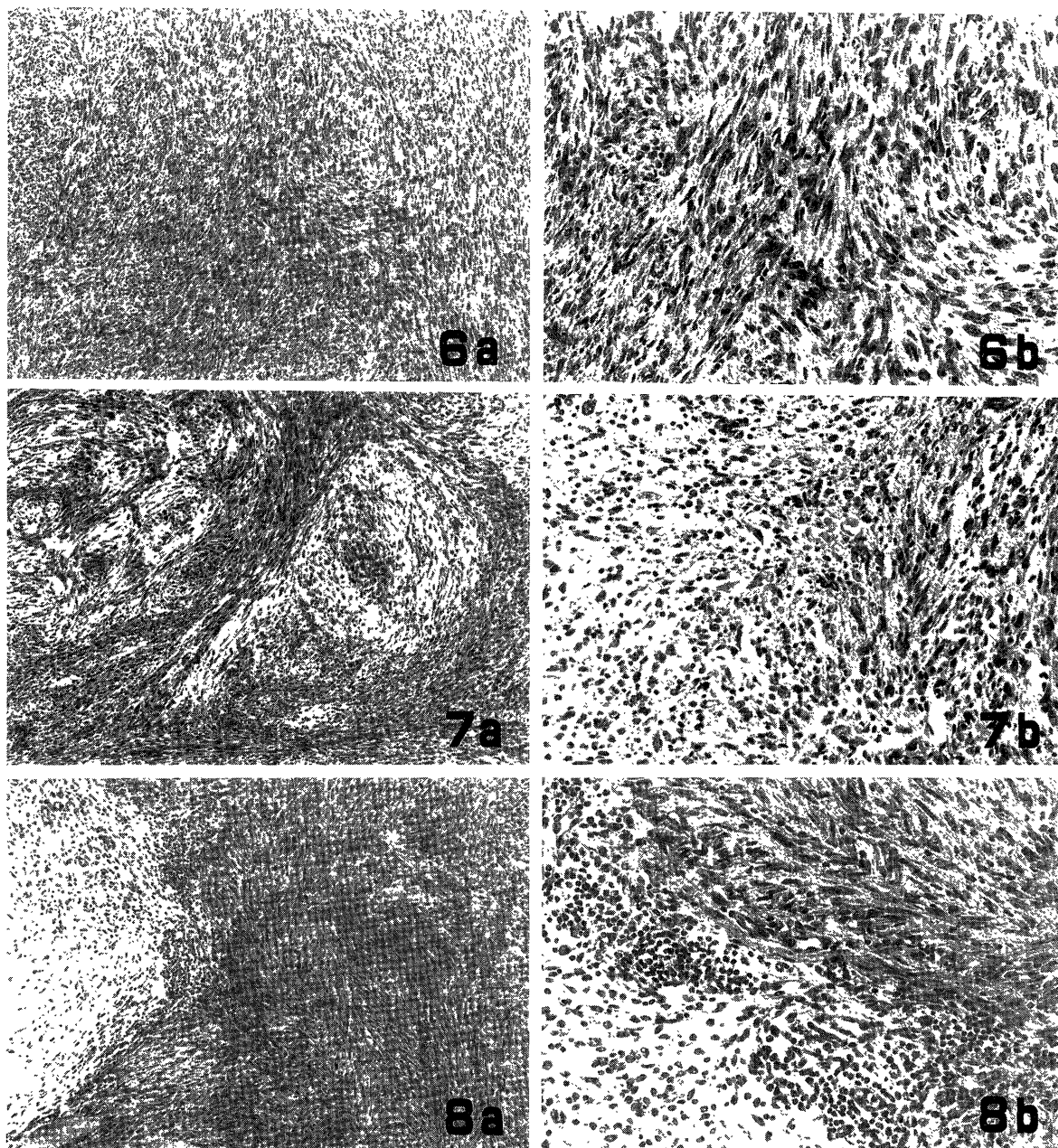


Fig. 6. Histological appearance of MC-induced tumor of control animal (No. I). Tumor cells of spindle type are arranged in bundles or a knot-like pattern (6a,  $\times 80$ ). Nuclei are rich chromatin and contain one or two prominent nucleoli (6b,  $\times 200$ ).

Fig. 7. MC-induced tumor of the rat treated with TLA (No. II) fallen into focal necrosis (7a,  $\times 80$ ). Infiltration of lymphocytes and neutrophils were observed at the inner side of residual tumor cells (7b,  $\times 200$ ).

Fig. 8. Central necrosis was enlarged in case of No. III whereas viable tumor cells were still resident (8a,  $\times 80$ ). Prominent cell infiltration and degenerative change of tumor cells were also observed (8b,  $\times 200$ ).

ably high level of LDH.

Histopathological studies revealed dense growths of spindle tumor cells in untreated control rats, while enlarged central necrosis with the infiltration of lymphocytes and neutrophils was apparent in

TLA-treated rats. Of particular interest was the presence of large granular Thy-1 positive cells in TLA-treated rats that were not observed in the untreated control group. Immunohistochemical examination demonstrated that these cells were occa-

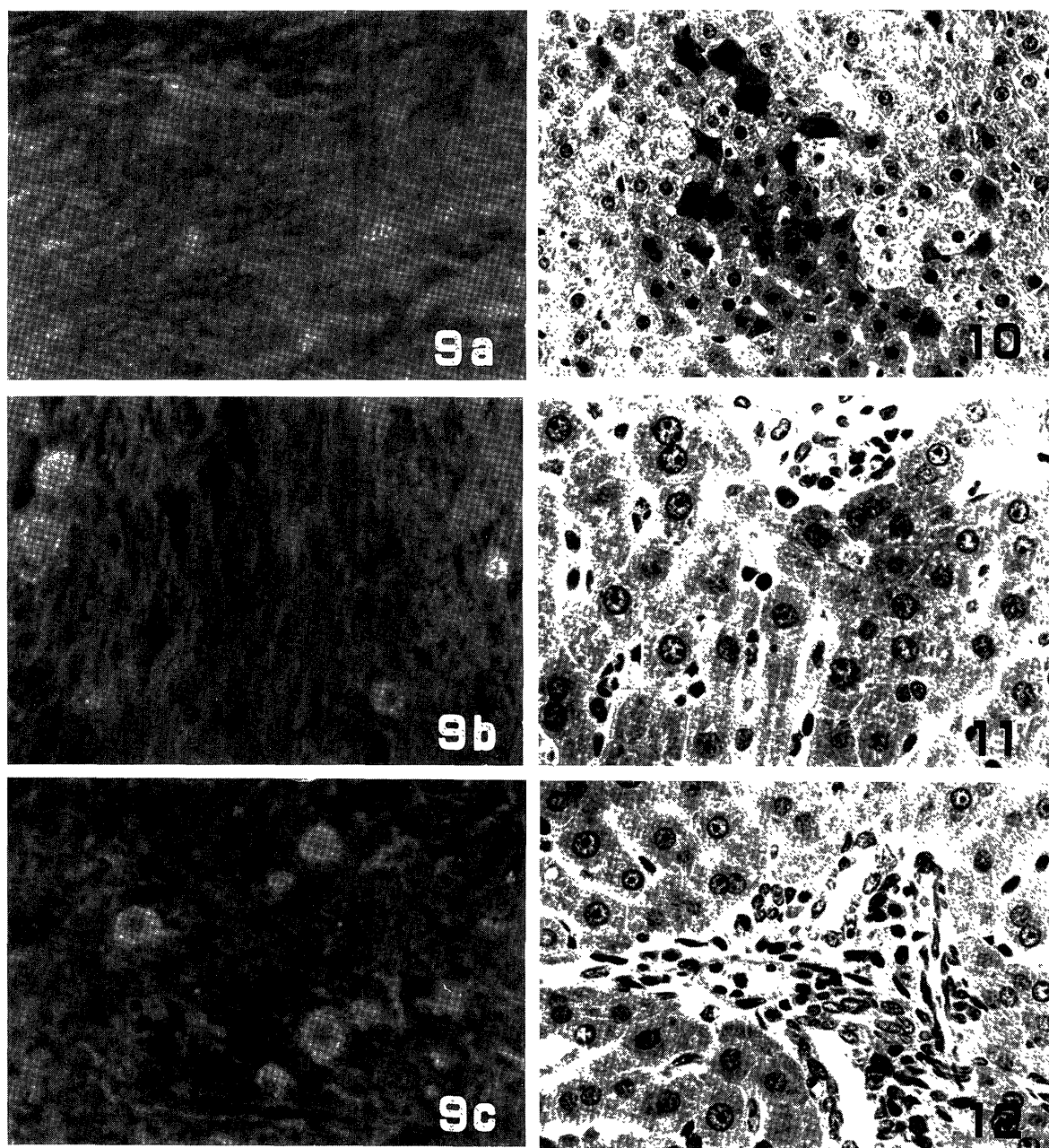


Fig. 9. Immunofluorescent staining of MC-induced tumor with FITC-labelled anti-rat Thy-1 mouse serum. A few small Thy-1 positive cells are present in the tumor tissue of the control animal, No. I (9a,  $\times 350$ ). In the tumor tissues of TLA-treated animals, No. II (9b) or No. III (9c), large granular labelled cells were detected sporadically.

Fig. 10. Histological appearance of the liver of control animal, No. I ( $\times 300$ ). Note the focal necrosis with cloudy swelling and pyknosis of liver cells.

Fig. 11. Interstitial infiltration of neutrophils, lymphocytes and large mononuclear cells were prominent in case of No. II ( $\times 500$ ).

Fig. 12. Degenerative changes of liver cells were moderate and cell infiltrations were observed in case of No. III as well as No. II ( $\times 500$ ). Swelling of arteriolar endothelial cells was also observed.

sionally positive for markers for non-helper T-cells. Authors have reported that incubation of spleen cells from TLA-sensitized mice with TLA caused an increase in the numbers of large Thy-1.2 positive cells and asialo GM<sub>1</sub> positive cells. These cells are highly cytotoxic to P-815 target cells (nonsensitive to NK cells) and YAC-1 target cells (sensitive to NK cells) [4, 12]. Results of this study suggest that large granular Thy-1 positive cells observed in tumor tissue might be identical to the cells reported earlier [6] and might participate in the inhibition of tumor growth.

Histological section of the liver of untreated tumor-bearing rat showed focal necrosis where severe degenerative changes such as cloudy swelling and pyknosis were prominent. The livers of TLA-treated rats seemed more vivid than control animals while moderate degenerative changes of liver cells were still observed. Intensive interstitial infiltrations composed of many neutrophils, a few lymphocytes and large mononuclear cells were observed in cases of TLA-treated rats. Previous studies have indicated that counts of sIg(+), Thy-1.2(+), Lyt-1.2(+), and asialo GM<sub>1</sub> (+) cells in the liver and peripheral blood of TLA-sensitized mice are likely to be higher than those of non-sensitized animals [4]. The cellular infiltrates observed in this study may correspond to the increases in specific cell populations that were observed in TLA-sensitized mice above mentioned. The *in vivo* effects of TLA on MC-induced tumors indicate that this substance may be a useful modifier of biological responses to tumor growth.

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