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Original Article

SST-2 Tumor Inoculation is a Useful Model for Studying the Anti-Tumor Immune Response in SHR Rats

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Abstract

Objective: The purpose of the present study was to investigate the relation between the dose of tumor cell inoculation (especially the doses less than minimum required to evoke tumor growth) and the anti-tumor immune system, particularly lymphoblast formation and cytotoxic activity of lymphocytes.

Method: We inoculated rats with various doses of SST-2 tumor cells and examined natural killer (NK) cell activity and lymphoblast formation *in vitro*.

Result: The results showed that the cytotoxicities against SST-2 cells and lymphoblast formation of lymphocytes were enhanced by small dose inoculation of tumor cells that could not induce tumor growth.

Conclusion: It was suggested that was lymphocutes play an important role as an anti-tumor immune system at small doses of tumor inoculation, which appears to reflect an early stage of tumor growth *in vivo*. It was also suggested that SST-2 tumor inoculation might be a useful model for studying the anti-tumor immune response in SHR rats.

Key words: SST-2, SHR rat, tumor inoculation, NK cell, T cell

Introduction

Cytotoxic T cells, natural killer (NK) cells and macrophages play important roles in anti-tumor immunity. In particular, NK cells were suggested to suppress tumor growth. However, it was reported that the number of these immune cells and the strength of their response are diminished in accordance with cancer progression (1–3).

Very small dose inoculation of tumor cells does not result in tumor growth. Most studies on the anti-tumor immune system utilize minimum tumor inoculation dose, which result in tumor growth. MHC class I molecules are expressed on the tumor cells at the early stage of tumor growth. Cytotoxic T cells recognize these molecules and mount an attack against the tumor (4). However, the small dose of tumor inoculation can not elicite NK cell cytotoxicity as a result of the expression of MHC class I molecules (5). It appears, therefore, that a thorough understanding of the immune response to small doses of tumor inoculation that are less than the minimum dose required to evolve tumor growth is necessary for a complete description of tumor growth.

Studies on the anti-tumor immune system that used allogeneic tumor cells for inoculation have reported instances of transplantation rejection (6). Therefore, the changes in the immune system induced by syngeneic tumor cell inoculation may be investigated to obtain a complete picture of the anti-tumor immune system in living bodies affected with cancer.

In the present study, spontaneously hypertensive rats (SHR) were inoculated with various doses of SST-2 tumor cells obtained from animals with mammary cancer. The purpose of the present study was to investigate the relation between the dose of tumor cell inoculation, especially doses less than the minimum required to evoke tumor growth, and the anti-tumor immune system, particularly lymphoblast formation and NK cell cytotoxic activity.

Materials and Methods

Animals

SPF inbred SHR female rats aged 4 months were obtained

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from Japan SLC, Inc. All rats were acclimatized in plastic cages for one week in a semi-barrier room under controlled environmental conditions (25±2°C, 55±10% humidity, 12-hour light-dark cycle). After one week of acclimatization, the rats were divided into 7 groups. There were 8 rats in each group. The average body weight in each group was 210±10 g at the start of the experiment. Rats were given autoclaved food (EQ#5L65; Japan SLC, Inc., Tokyo, Japan) and distilled water *ad libitum* throughout the experiment.

Tumor cells inoculation

The tumor cells (SST-2) that originated from spontaneous mammary adenocarcinoma in SHR animals were provided by Professor Moriguchi (Department of Nutrition, Yamaguchi Prefecture University, Yamaguchi, Japan). The tumor cells were placed in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Japan) containing 7.4% fetal bovine serum (FBS: MBL Co., Ltd., Tokyo, Japan) and incubated at 37C in 5% CO_2. The cells were released by 0.25% trypsin with 0.02% EDTA, and the cell number was adjusted to 5×10^5 cells/ml (Group 1), 1×10^6 cells/m (Group 2), 2×10^6 cells/ml (Group 3), 5×10^6 cells/ml (Group 4), 1×10^7 cells/ml (Group 5) and 2×10^7 cells/ml (Group 6) in saline. One-hundred microliters of cell suspension at each dose was subcutaneously inoculated into the back of the rats. The control animals were inoculated with saline. Two weeks after inoculation, the tumors were removed from the back of the rats and weighed.

Peripheral blood leukocyte distribution

Blood was collected while the animals were anesthetized with Nembutal (Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan). The number of leukocytes was then counted using an automatic blood cell counter (Sysmex F-820, Toa Medical Electron Inc., Tokyo, Japan). Leukocyte classification was performed using May-Giemsa staining.

Lymphocyte blasted formation test

The lymphoblast formation (LBF) was measured by using splenic cells. The spleen was removed from rats under sterile conditions, minced in medium, and mashed with glass slides. The crude cell suspension was then filtered through a nylon mesh and suspended in RPMI 1640 containing 7.4% FBS. Lymphocytes were separated by Lympholyte Rat (Cedarlane Lab., Ltd., Ontario, Canada) and adjusted to a final concentration in 3×10^5 cells/ml of RPMI 1640 medium containing 7.4% FBS. The splenic lymphocyte suspension was cultured for 72 hours at 37°C in 5% CO_2 in an incubator. The cells were activated using the following mitogens: concanavalin A (ConA; 0.125 μg/ml; Sigma Chemical Co., St. Louis, MO), phytohemagglutinin (PHA; 1 μg/ml; Sigma Chemical Co., St. Louis, MO), lipopolysaccharide (LPS; 0.53 μg/ml; Sigma Chemical Co., St. Louis, MO), and pokeweed mitogen (PWM; 0.26 μg/ml; Sigma Chemical Co., St. Louis, MO). After the incubation, the absorbance of each suspension was measured using a Cell Counting Kit (Dojindo Lab., Kumamoto, Japan). The LBF was calculated using the following formula: Activity (%)=[(Stimulation Test-Spontaneous)/Spontaneous]×100.

Cytotoxic activity assay

Splenic lymphocytes were isolated in the same manner as for the LBF test. Suspended splenolymphocytes at a concentration in 5×10^6 cells/ml were used as the effector cells. YAC-1 cells obtained using the standard method and the SST-2 inoculated tumor cells used in this experiment were used as target cells. The target cells were adjusted to a concentration in 5×10^4 cells/ml in RPMI 1640 containing 7.4% FBS. One-hundred microliters of effector and target cell suspensions were co-cultured on a microplate. The ratio of effector cells to target cells was 100:1. The killing activity of the splenic lymphocytes was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega Co. Madison, WI). The LDH release assay is based on the same principle as the ^{51}Cr release assay (7, 8). The results were expressed as the percentage of specific release, i.e., percentage of cytotoxicity, and were determined using the following formula: %Cytotoxicity=[(Experimental-Effector Spontaneous-Target Spontaneous)/(Target Maximum-Target Spontaneous)]×100.

Statistical analysis

The difference in the inter-group comparisons was analysed using one-way factorial analysis of variance (ANOVA). The above data tested for significance by ANOVA were analyzed post hoc with Fisher's PLSD test.

Results

Tumor growth

All rats inoculated with various doses of tumor cells survived the term of the experiment. No significant difference in body weight was observed among the groups (data not shown). However, the wet weights of the SST-2 tumors obtained from the backs of the rats two weeks after the inoculation differed among the 7 groups. In particular, the weight of the SST-2 tumors increased with the dose of inoculated tumor cells. The growth of the SST-2 tumor was maintained at low levels in

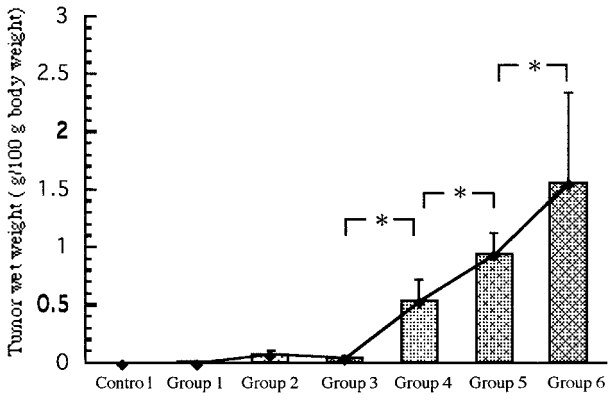

Fig. 1 Dose effect of SST-2 inoculation on the tumor growth in SHR rats. Rats were subcutaneously inoculated 5×10^4, 1×10^5, 2×10^5, 5×10^5, 1×10^6, and 2×10^6 of SST-2 tumor cells, in Group 1, Group 2, Group 3, Group 4, Group 5, and Group 6, respectively. There were 8 rats in each group. After 2 weeks, the fixed tumor was weighted, and the weight was expressed per 100 g body weight. Wet weight of the tumor was expressed as mean±SE. *Indicates significant difference between groups (Fisher's post hoc test, $p<0.05$).

Table 1 Hematological parameters of SHR rats inoculated with SST-2 tumor cells

Group	Control	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Total leukocytes ($10^2/\mu l$)	54.0±20.8	59.0±24.7	69.0±20.6	45.0±14.1	69.0±18.4	77.0±18.1*	72.0±45.0
Lymphocytes ($10^2/\mu l$)	32.0±13.9	36.4±12.4	40.8±15.1	24.8±7.8*	35.0±12.6	29.7±8.4	22.9±7.9
Neutrophils ($10^2/\mu l$)	12.2±4.2	12.5±9.9	20.1±10.3	13.3±6.7	35.7±19.8†	40.4±14.0†	40.8±30.7†
Monocytes ($10^2/\mu l$)	8.3±4.2	9.0±2.9	8.3±1.9	6.1±2.6	10.4±4.9*	9.3±3.7	6.3±3.3
Eosinophils ($10^2/\mu l$)	1.2±0.7	1.2±0.8	0.5±0.5	0.8±0.6	1.5±1.0	1.6±1.6	0.5±0.7

Values are means±SE. Significant difference was observed in lymphocyte numbers between Group 2 and Group 3. In other cases, significant differences compared with the value of the control group are indicated as * $p<0.05$, † $p<0.01$ (Fisher's post hoc test).

Groups 1–3 (Fig. 1). However, tumor growth was observed when the SST-2 cells were inoculated more than 2×10^5 cells/rat.

Peripheral blood leukocytes

Table 1 shows the leukocyte distribution in peripheral blood. The total leukocyte count showed a tendency which increase in accordance with a dose increase. This tendency coincided with an increase in neutrophils ($p<0.01$). On the other hand, the number of lymphocytes decreased in Group 3 compared with that of the Group 2. Significant changes were not observed in other leukocytes. These results show that the increase in total leukocytes seen with larger inoculation doses mostly reflects increases in the number of neutrophils.

Lymphocytes blast formation (LBF)

Four types of mitogens were used in this experiment. Figure 2 shows the rate of lymphocyte blast formation in the splenolymphocytes obtained from the inoculated rats. A marked increase in the rate of blastogenesis was observed in Groups 1 and 2 ($p<0.05$) except for the PWM treatment. However, the rate of blastogenesis in Groups 3–7 was returned to the control levels in any stimulation.

Cytotoxicity against SST-2

Figure 3 (A) shows the cytotoxic activity of lymphocytes (CAL) against the SST-2 cells used in the tumor inoculation. As a result of the small dose inoculation which could not induce the tumor growth (Group 1), The CAL against SST-2 was significantly increased ($p<0.05$). However, the CAL was return to the control levels at minimum dose inoculation that was required for tumor growth (Group 3). Then, a slight increase in CAL was observed in Groups 4 and 5 ($p<0.1$). When the size of the tumor inoculation further increased (Group 6), CAL against SST-2 decreased again.

Cytotoxicity against YAC-1

CAL against YAC-1 showed a different tendency at the small dose of inoculation, which could not induce the tumor growth, compared with that against the SST-2 (Fig. 3). No difference in the cytotoxicity was observed between Groups 1 and 2. In addition, the cytotoxicity was lowest at the minimum dose inoculation required for tumor growth (Group 3) similar to the case of the SST-2 inoculations. Then, a slight increase in CAL was observed in Groups 4 and 5. When the size of the tumor inoculation further increased (Group 6), CAL against YAC-1 decreased again. In the control group, CAL against YAC-1 was higher than that against SST-2.

Discussion

In this study, tumor growth accompanied the inoculation size when the rats were inoculated more than 2×10^5 cells/rat (Fig. 1). This shows that the minimum size required for tumor growth is 2×10^5 cells/rat, which agrees previous findings (9). Thus, a population of 2×10^5 cells/rat represents a critical point in the growth of SST-2 tumors, and the anti-tumor immune response against SST-2 might be changed at this point.

In the present study, the LBF to PHA and LPS was significantly enhanced at inoculation doses of less than 2×10^5 cells/rat (Fig. 2). However, no significant enhancement in the LBF to all mitogens was observed at the dose of 2×10^5 cells/rat or greater (Fig. 2). The presernt results suggest that T cells play an important role for the anti-tumor defense against SST-2 cells at least in small inoculation doses, although Matsuoka et al. (10) reported that the participation of NK cells is a principal effector mechanism in the suppression of SST-2 tumor growth in SHR rats.

In general, cytotoxic T cells play an important role in the anti-tumor immune system by recognizing MHC class I molecules which are expressed in tumor cells during the early stage of tumor growth (4). As the expression levels of MHC class I molecules are reduced with accompanied tumor growth, the tumor cells can evade the T-cell response (11). Moreover, tumor cells can escape the cellular immune response by the production of factors that inhibit lymphocyte proliferation (12). Thus, in the present, it appears that rats inoculated greater than 2×10^5 SST-2 cells could not prevent the tumor growth that resulted from the reduced activity of T cells.

It is interesting that, in rats inoculated 2×10^5 cells/rat, the number of peripheral lymphocytes was decreased (Table 1) as well as the proliferation activity of lymphocytes against LPS (Fig. 2) compared with rats inoculated with 1×10^5 cells/rat. Although the LBF to LPS showed a B-cell response, it appears that B cells do not play a direct role in the anti-tumor immune system. The increase in the B-cell response might be induced by the T-cell response to the tumor inoculation.

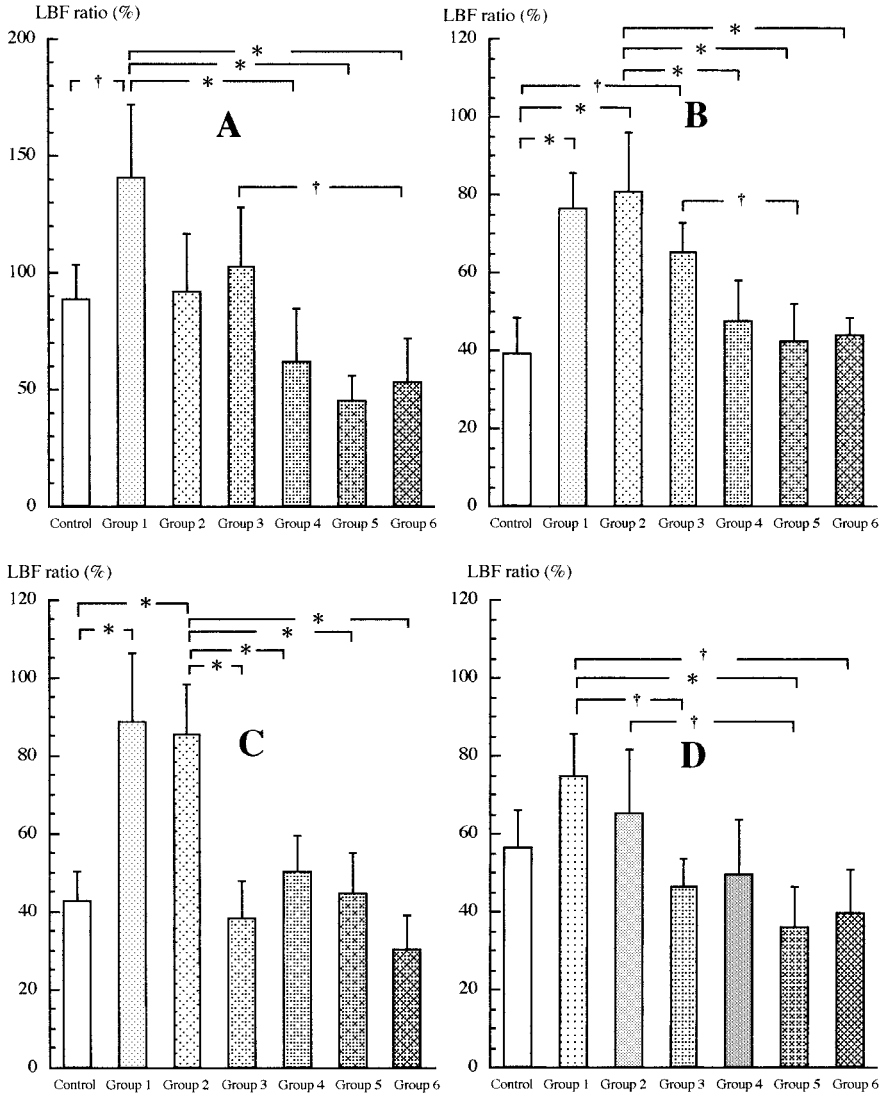

Fig. 2 Lymphocyte blast formation response (LBF) is enhanced by the small dose of SST-2 inoculation to SHR rats. Splenic lymphocytes were stimulated by Con A; 0.125 μg/ml (A), PHA; 1 μg/ml (B), LPS; 0.53 μg/ml (C), and PWM; 0.26 μg/ml (D) for 72 hours. LBF is expressed as mean±SE. * Indicates significant difference between groups (Fisher's post hoc test, $p<0.05$). † Indicates significant difference between groups (Fisher's post hoc test, $p<0.1$).

The cytotoxic activity (including the activities of NK cells and cytotoxic T cells) against SST-2 cells was increased significantly when the inoculation dose was 5×10^4 cells/rat which could not induce the tumor growth (Fig. 1), although the activity against YAC-1 cells was not increased at this inoculation size. Conceivably, the increase in CAL (an anti-tumor immune response) may be induced by the reduction in MHC class I molecule expression that occurs with tumor growth (13).

The present results showed that the change in CAL according to the tumor inoculation was varied depending on the target cell types. Cytotoxicity against SST-2 cells was enhanced at small doses of inoculation, but the activity against YAC-1 cells was not. The difference in the response of CAL might indicate a difference in the characteristics between SST-2 and YAC-1 cells. Since YAC-1 cells are known to be relatively more sensitive to NK cells than other cell lines, YAC-1 cells are commonly used as the target cells for measuring NK cytotoxicity (14, 15). In fact, in the control group, the splenic lymphocyte cytotoxicity against YAC-1 cells was clearly higher than that against SST-2 cells (Fig. 3). It appears that the syngenesis and the low (not too high) sensitivity of SST-2 cells to NK cell cytotoxicity make it possible to reflect the *in vivo* state of anti-tumor NK-cell responses.

Investigation of the immune response at small tumor inoculation dose appears very important to the elucidation of anti-tumor immune defense mechanisms. Furthermore, the use of syngeneic tumor cells in addition to the use of YAC-1 cells for the measurement of lymphocyte cytotoxic activity provides useful information about immune defense systems during the early stage of tumor growth.

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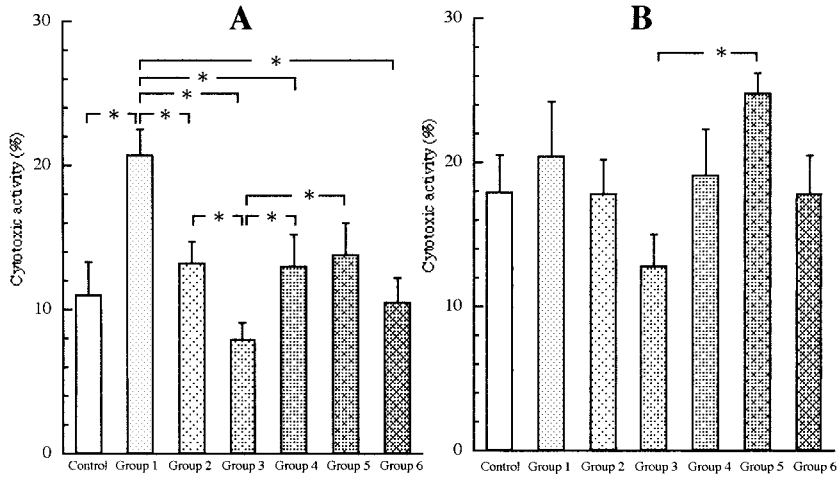

Fig. 3 Small dose of SST-2 inoculation enhances cytotoxic activity of lymphocyte against SST-2 cells but not against YAC-1 cells. ***In vitro*** **killing activity of splenic lymphocyte against SST-2 cells (A) and YAC-1 cells (B) was measured by LDH release assay. In both target cells, the activity was lowest in Group 3 that was the minimum dose required for the tumor growth (Fig. 1), and the activity was increased in Group 5 compared with Group 3. In small inoculation doses (Group 1), the activity was enhanced only against the SST-2 but not against the YAC-1. Cytotoxic activity is expressed as mean±SE. * Indicates significant difference between groups (Fisher's post hoc test, $p<0.05$). † Indicates significant difference between groups (Fisher's post hoc test, $p<0.1$).**

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