Jpn. J. Cancer Res. 81, 253–258, March 1990

# Cyclophosphamide-dependent Lymph Node Modification in Lymph Node Metastasis of MM48 Tumor Cells in Syngeneic Mice

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**We investigated the role of immunosuppressive activity induced in the regional lymph nodes (RLN, popliteal lymph nodes) in the establishment of lymph node metastasis by cyclophosphamide (CY) administration. The CY treatment led to the elimination of suppressive activity with the appearance of positive immune responses, and the inhibition of lymph node metastasis of MM48 tumor cells. In CY-treated mice, the removal of RLN together with the primary tumor lowered the survival rate compared with the mice in which the RLN remained intact. During 4 days after primary tumor resection, the proliferation of tumor cells in the RLN was significantly decreased in CY-treated mice. These results suggested that the induction of suppressive activity in the lymph node is closely associated with the establishment of lymph node metastasis.**

Key words: Lymph node metastasis — Suppressive activity —Cyclophosphamide

One of the most difficult problems confronting contemporary cancer therapy is the prevention of secondary spread of tumor cells by appropriate means. We have investigated the relationship between immune responses and the proliferation of tumor cells in the regional lymph nodes (RLN) using highly metastatic MM48 tumor of C3H/He mice. In this tumor system, immune responses such as immunoregulatory activities are induced only in RLN, but not in systemic lymphoid organs (spleen and thymus). Therefore, this model is distinctive and has the advantage of allowing analysis of the role of regional lymph nodes in metastasis without the influence of the systemic immunity.[1,2] It was demonstrated that the growth of migrant tumor cells in RLN is relevant to the immunoregulatory activities induced in RLN, and Lyt-1-positive suppressor T cells induced in RLN play a crucial role in the establishment of lymph node metastasis. These results suggest that the elimination of suppressor T cells by appropriate means would prevent lymph node metastasis.

In this study, the experiments were designed to determine the effect of CY[3–7] administered just after the appearance of suppressor T cells on immune status and metastasis in the RLN.

## MATERIALS AND METHODS

**Animals** For all the experiments specific-pathogen-free female inbred C3H/HeSlc mice at 6 weeks of age were purchased from SLC (Hamamatsu) and were used at 8–10 weeks old for the experiments.

Abbreviations used: CY, cyclophosphamide; PEC, peritoneal exudate cells; PBS, phosphate-buffered saline; RLN, regional lymph node (popliteal); MEM, minimum essential medium.

**Tumors** A transplantable ascites mouse mammary tumor MM48 cell line was used. It was established from a spontaneous mammary tumor of murine mammary tumor virus-positive C3H/He mouse and is moderately immunogenic. MM48 tumor cells form a solid tumor when an appropriate number of tumor cells are injected subcutaneously and cause metastases predominantly to lymph nodes. Tumor cells were maintained by serial intraperitoneal transplantation into syngeneic mice.

**Chemotherapy** CY (Endoxan, Shionogi Pharmaceutical Co., Ltd., Osaka) was dissolved in sterile PBS. An optimal dose of CY (70 mg/kg) was inoculated intraperitoneally on days 13 and 14 of tumor growth, corresponding to the early phase of suppressor T cell emergence in the RLN.[2]

**Preparation of lymphocytes** Mice were immunized with $2 \times 10^6$ irradiated tumor cells (100 Gy, $^{60}$Co source) once a week for three weeks. Ten to 11 days after the last immunization, the mice were killed and peritoneal exudate cells (PEC) were obtained. PEC were passed through a Sephadex G-10 column to obtain a lymphocyte-enriched fraction. Eighty-five to ninety percent of the Sephadex G-10 passed fraction of PEC was Thy-1-positive. Lyt-2-positive T cells showed cytotoxic activity and were used as effector cells in the assays for immunoregulatory activity.

Lymph nodes were removed aseptically and were minced to make a single cell suspension in RPMI1640 medium supplemented with 10% fetal calf serum, 10 m*M* HEPES, penicillin and streptomycin.

**Antibodies used** Anti-Thy-1.2 antiserum was produced in AKR mice by intraperitoneal injections of C3H thymus cells in our laboratory. Monoclonal anti-Lyt-1.1 and anti-Lyt-2.1 antibodies were obtained commercially

(Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada).

**Cytotoxicity test** One million effector cells were incubated with $10^4$ target cells labeled with $^{51}Cr$ (sodium chromate, New England Nuclear, Boston, MA) in 0.2 ml of Dulbecco's MEM supplemented with 10% fetal calf serum, 10 m*M* HEPES, penicillin and streptomycin. The assay was run in a culture plate with U-bottomed wells (Microtest plate, Nunc, Roskilde, Denmark) with 5% $CO_2$ in air at 37°C. After 12 h incubation, 0.1 ml of the supernatant was removed and radioactivity was measured on a gamma counter (Auto Gamma 500C; Packard Instrument Co., La Grange, IL). The cytotoxicity was calculated as follows; % specific $^{51}Cr$ release = [test release (cpm) − control release (cpm)]/[maximum release (cpm) − control release (cpm)] × 100.

**Assay for immunoregulatory activity** The experimental procedure used to assay the activity of immunoregulatory cells was a slight modification[1,2] of the method described by Yamauchi *et al.*[8] Briefly, lymphoid cells to be tested were added to a mixture of effector and target cells at a target:effector:regulatory cell ratio of 1:150:150. The plate was incubated with 5% $CO_2$ in air at 37°C for 12 h and radioactivity released in the supernatant was measured.

**Operation of mice** In mice under pentobarbital anesthesia, the tumor-bearing leg was amputated after ligation just below the knee joint. The proximal cut-end was disinfected daily with 5% tincture of iodine for a week. The distal cut-end was stamped on a microslide glass. The specimen was stained with Wright-Giemsa staining solution and it was confirmed that no tumor cells remained at the cut-end.

**Examination of metastasis in the RLN by bioassay** The popliteal lymph node of tumor-bearing mice was removed aseptically and teased apart in PBS. Cell suspension equivalent to one lymph node, irrespective of organ size, was transferred intraperitoneally into a syngeneic naive mouse. Metastasis in the lymph node was judged positive when the recipient given the lymph node cells to be tested died of ascites tumor. One hundred MM48 tumor cells are a sufficient dose to kill 100% of syngeneic recipients, when inoculated intraperitoneally.

## RESULTS

**Effect of CY administration on lymph node metastasis** One million MM48 tumor cells were implanted subcutaneously into the hind footpad. On days 13 and 14, CY (70 mg/kg) was administered intraperitoneally and the primary tumor in the hind foodpad was resected on day 14. PBS was injected instead of CY in the control mice. The survival rate was compared among the groups of mice that received no operation (group A), resection of the primary tumor only (group B), resection of the primary tumor together with RLN removal (group C), CY-administration plus resection of only the primary tumor (group D), and CY-administration plus resection of the primary tumor together with RLN removal (group E) (Table I). Surgical operation alone, such as primary tumor resection or primary tumor resection together with RLN removal caused little increase in the survival rate (compare groups B and C with group A). However, CY administration significantly elevated the survival rate ($P<0.05$, chi-square test) when mice received only primary tumor resection (compare group D with group A). RLN removal in CY-treated mice resulted in a decrease of survival rate, though not significant, compared with the mice having intact RLN (compare group D and group E). The survival rate was recorded for 100 days. The survival time of mice that died in each group was as follows; group (A) 37.2±12.4 days, group (B) 40.1±9.8 days, group (C) 41.3±16.8 days, group (D) 68.9±20.3 days, group (E) 50.6±22.2 days. The survival time

Table I. Effect of CY Administration, Primary Tumor Resection and RLN Removal on the Survival Rate of Mice[a]

| Group | CY[b] | Resection of primary tumor[c] | Removal of lymph node[d] | Survival rate[e] | | | |
|---|---|---|---|---|---|---|---|
| | | | | Exp. 1 | Exp. 2 | Exp. 3 | Total |
| A | − | − | − | nd | 0/10 | nd | 0/10 |
| B | − | + | − | 1/10 | 1/10 | nd | 2/20 |
| C | − | + | + | 1/10 | 0/10 | nd | 1/20 |
| D | + | + | − | 5/10 | 4/10 | 5/9 | 14/29 |
| E | + | + | + | 1/10 | 3/10 | 2/8 | 6/28 |

*a*) One million MM48 tumor cells were implanted subcutaneously into the hind footpad on day 0.
*b*) + = 70 mg/kg of CY was administered intraperitoneally on days 13 and 14. − = not administered.
*c*) Performed on day 14.
*d*) Performed at the time of primary-tumor resection on day 14.
*e*) Recorded for 100 days. nd = not done.

Table II. Modification of Immunoregulatory Activity in the RLN by CY Administration[a]

| Popliteal node taken from[b] | CY[c] | Antibody treatment | Percent specific $^{51}$Cr release | Regulatory activity |
|---|---|---|---|---|
| Normal mouse | − | − | 8.7±0.7[d] | Control |
| Operated mouse | − | − | 0.9±0.1 | Suppressive[e] |
| Operated mouse | + | − | 14.2±0.7 | Augmentative |
| Operated mouse | + | Anti-Thy-1+C | 8.5±0.3 | − |
| Operated mouse | + | Anti-Lyt-1+C | 8.6±0.2 | − |
| Operated mouse | + | Anti-Lyt-2+C | 14.5±0.4 | Augmentative |

*a*) One million MM48 tumor cells were implanted subcutaneously into the hind footpad on day 0.
*b*) Lymphoid cells of popliteal lymph node were added to a mixture of effector and target cells as described in "Materials and Methods." The assays were performed 1 day after primary tumor resection (day 15).
*c*) +=70 mg/kg of CY was administered intraperitoneally on days 13 and 14. −=not administered. On day 14, the primary tumor was resected.
*d*) Values are mean±SEM of quadruplicate cultures in 3 independent experiments.
*e*) Significant increase and decrease in $^{51}$Cr release compared with the control by addition of test lymphoid cells were regarded as due to augmentative and suppressive activities, respectively ($P<0.05$, Student's $t$ test).

Table III. Appearance of Cytotoxic Activity in the RLN after CY Administration[a]

| Popliteal node taken from[b] | CY[c] | Antibody treatment | Percent specific $^{51}$Cr release | Cytotoxic activity |
|---|---|---|---|---|
| Normal mouse | − | − | 1.6±0.7[d] | Control |
| Operated mouse | − | − | −1.4±0.2 | − |
| Operated mouse | + | − | 6.4±0.4 | +[e] |
| Operated mouse | + | Anti-Thy-1+C | 0.6±1.2 | − |
| Operated mouse | + | Anti-Lyt-1+C | 6.8±0.4 | + |
| Operated mouse | + | Anti-Lyt-2+C | −1.6±0.2 | − |

*a*) One million MM48 tumor cells were implanted subcutaneously into the hind footpad on day 0.
*b*) Lymphoid cells of popliteal lymph node were added to $^{51}$Cr-labeled target cells as described in "Materials and Methods." The assays were performed 1 day after primary tumor resection (day 15).
*c*) +=70 mg/kg of CY was administered intraperitoneally on days 13 and 14. −=not administered. On day 14, the primary tumor was resected.
*d*) Values are mean±SEM of quadruplicate cultures in 3 independent experiments.
*e*) Significant ($P<0.05$, Student's $t$ test) compared with control.

showed the same tendency as the survival rate. On day 100, all the mice that had survived were autopsied and it was confirmed that they were free of tumor in all organs.

The cause of death was metastases in distant and regional lymph nodes. Sometimes, pulmonary metastasis was found at autopsy, but it was not so severe as to be fatal.

**Effect of CY administration on immune responses in the RLN** Immune responses in the RLN of mice that received primary tumor resection were examined in CY-treated and control groups. One million MM48 tumor cells were implanted subcutaneously into the hind footpad. On days 13 and 14, CY (70 mg/kg) was administered intraperitoneally and the primary tumor in the hind foodpad was resected on day 14. On day 15, mice were killed and lymphoid cells of the RLN were assayed for immunoregulatory and cytotoxic activities. PBS was injected instead of CY in the control mice.

Immunoregulatory activities in the RLN were assayed using MM48-specific cytotoxic T cells in the PEC of intraperitoneally immunized mice as effector cells (Table II). Lymphoid cells to be tested for immunoregulatory activity were added to a mixture of effector and target cells. Killer-augmenting and suppressive activities for cytotoxicity of immune T cells were assessed on the basis of a significant increase or decrease of $^{51}$Cr release com-

Table IV. Effect of CY Administration on RLN Metastasis[a]

| RLN taken from | CY[b] | Take incidence of tumor | | | |
|---|---|---|---|---|---|
| | | 6 h[c] | Significance[d] | 4 days[c] | Significance[d] |
| Unoperated | − | 10/10 | Control | 10/10 | Control |
| Operated[e] | − | 19/20 | NS | 18/20 | NS |
| Operated | + | 18/20 | NS | 10/20 | Significant |

*a*) One million MM48 tumor cells were implanted subcutaneously into the hind footpad on day 0.
*b*) +=70 mg/kg of CY was administered intraperitoneally on days 13 and 14. −=not administered.
*c*) Six hours or 4 days after tumor resection, a single-cell suspension of one RLN per mouse was transferred into syngeneic naive mice. Tumor take was recorded for 40 days.
*d*) Compared with the control. Significant ($P<0.05$, chi-square test). NS=not significant.
*e*) Performed on day 14.

pared with the control during 12 h incubation, respectively. Suppressive activity was eliminated by CY administration and killer-augmenting activity appeared in the RLN. The killer-augmenting activity was abrogated by treatment of RLN cells with anti-Lyt-1.1 antibody plus complement. In addition, cytotoxic activity to MM48 tumor cells appeared in the RLN of CY-treated mice (Table III). The cytotoxic activity was abrogated by treatment with anti-Lyt-2.1 antibody plus complement, but not by treatment with anti-Lyt-1.1 antibody plus complement.

Killer-augmenting and cytotoxic activities were not found in distant lymph nodes such as lumbar, inguinal or axillary lymph nodes of the ipsilateral side. As immune responses (such as immunoregulatory activity or cytotoxic activity) were not detectable in spleen or thymus,[2] the effect of CY administration was not investigated in these systemic lymphoid organs.

**Effect of CY administration on RLN metastases examined by bioassay** The RLN in mice that received CY administration before primary tumor resection seemed to play a beneficial role for the host. Why do the migrant tumor cells not grow to form metastatic foci? The proliferation of migrant tumor cells in the RLN was examined by transferring RLN cells into naive mice (Table IV). CY was given to tumor-bearing mice on days 13 and 14, and on day 14, the primary tumor was resected. After various intervals, a single-cell suspension of the RLN was transferred intraperitoneally to naive mice. In the assay performed 6 h after primary tumor resection, there was no difference in the tumor take incidence between CY-treated mice and the mice without tumor resection. However, in the assay performed 4 days after operation, CY administration decreased the take incidence significantly ($P<0.05$, chi-square test). In operated mice without CY administration, no decrease was observed in the take incidence.

## DISCUSSION

In a tumor system capable of stimulating both the peripheral and central lymphoid organs, the immunological activity of the lymph node will be influenced by systemic immunity manifested in the spleen. In this respect, our MM48 tumor model is distinctive and has the advantage of allowing analysis of the role of the RLN in metastasis without the influence of specific immunity evoked by the spleen. In our previous reports,[1,2] immunoregulatory activity in the RLN was evaluated in terms of metastasis to the RLN during the development of the primary tumor. Killer-augmenting activity appeared in the early phase of tumor growth and showed its highest activity on day 10. Thereafter, suppressive activity emerged and the detection rate of tumor cells in the RLN increased. This phase was followed by tumor proliferation in the RLN detected by microscopic observation. In the experiments using locally preimmunized mice, it was suggested that the induction of suppressive T cell in RLN played a crucial role in the establishment of metastasis. It was proposed that elimination of suppressive activity by appropriate means might alter the immune status in the RLN and lead to inhibition of the secondary spread of tumor cells.

CY is a non-specific alkylating agent which acts upon actively dividing cells and has been used as a chemo-immunotherapy agent to eliminate suppressor T cells.[9-11] In CY-treated mice, suppressive activity which arose in the RLN of mice without CY-administration was eliminated, and the two types of effector cells involved in positive immune responses (killer-augmenting T cells with Lyt-$1^+$ $2^-$ and cytotoxic T cells with Lyt-$1^-$ $2^+$) became dominant in the RLN (Tables II and III). Such a change of immune status prevented tumor proliferation in the RLN (Table IV) and led to an increase in the survival rate (Table I). CY itself had no effect on

the viability of migrant tumor cells in the RLN, because tumor cells in the RLN of CY-treated mice grew as well as those of control mice in the bioassay performed 6 h after tumor resection. But, the tumor take was significantly lowered in the assay 4 days after the operation (Table IV). In addition, administration of CY at a larger dose did not have a greater effect than an optimal dose did (data not shown). It is probable that some mechanisms such as positive immune response emerged and participated in the elimination or inhibition of tumor proliferation in the RLN during 4 days.

Alternatively, a certain change of immune status in the RLN might alter the tumor-trapping capacity of RLN. Hewitt and Blake[12] have demonstrated that tumor cells pass through the nodes but do not accumulate or proliferate in a non-immunogenic tumor system. MM48 tumor is not non-immunogenic, but is moderately immunogenic. However, in the immunoattenuated status induced by CY administration, MM48 tumor might act as if it were nonimmunogenic. It is possible that the low incidence of tumor take in bioassay of the RLN was due to the small number of tumor cells accumulated in the RLN. It is still unclear whether migrant tumor cells were destroyed in the RLN or whether potent tumor cells passed through the RLN and reached the secondary or tertiary lymph nodes. The latter may be more probable. In CY-treated mice, nearly half the mice survived without any sign of tumor when they received only primary tumor resection (Table I). Half the mice died of metastases in distant lymph nodes, such as inguinal, axillary and lumbar lymph nodes. Metastasis in the remaining RLN (popliteal) was rare. However, the T-cell enriched fraction of the RLN showed no neutralizing activity *in vivo* (data not shown).

Cytotoxic T cells (Lyt-1$^-$ 2$^+$) appearing in the RLN should supplement $^{51}$Cr release by cytotoxic T cells of PEC in the assay for immunoregulatory activity. But, the increase of $^{51}$Cr release was abrogated by treatment of RLN cells with anti-Lyt-1.1 antibody plus complement (Table II). The reason why an additive increase of $^{51}$Cr release was not observed in the immunoregulatory assay is not known. Other mechanisms such as delayed-type hypersensitivity might operate in the augmentation of cytolysis. Lyt-2-positive T cells did not show activity in neutralizing assay *in vivo*, but L3T4-positive T cells were potent effectors in Winn's assay (data not shown). The immunoregulatory assay using cytotoxic T cells as an effector might not be adequate to establish the precise mechanism of *in vivo* immune responses against tumor cells in the RLN.

The resection of the primary tumor at an early stage of tumor development was prerequisite to avoid induction of the suppressive activity[13, 14] as well as to prevent tumor cell migration. However, little increase in inhibitory effect on secondary spread of tumor cells was obtained only by surgical treatments. CY administration modified the anti-tumor activity in lymph nodes, and raised positive immune responses, which had been down-regulated by suppressive activity[15] in the RLN. These factors should contribute to some extent to prevention of the secondary proliferation of migrant tumor cells in the RLN.[16]

## ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture in Japan.

*(Received October 6, 1989/Accepted January 13, 1990)*

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