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Abstract

Eradication of immunologically-syngeneic tumors was achieved by adoptive chemotherapy using effector cells induced by Corynebacterium parvum-Pyridine Extract Residue (CP-PER). A mixture of 2 X 10(6) Meth A cells and 0.1 mg CP-PER was subcutaneously inoculated into the back of donor BALB/c mice, with the result that their spleen cells showed an antitumor effect 10 to 13 days after the inoculation. These cells were used as immune cells. Recipient mice were inoculated with 1 X 10(6) Meth A cells, and 2 days later were administered cyclophosphamide. On the following day, 1 X 10(8) immune cells were adoptively transferred into the recipient mice. As a result, the tumor began to regress 7 to 12 days after the adoptive transfer. An immuno-histochemical study of the donors' spleens and the recipients' regressing tumors revealed that the ratio of L3T4+ T cells to Lyt-2+ T cells in the donors' spleens was increased and that the infiltrating cells in the recipients' tumors were mainly composed of L3T4+ T cells. This confirmed that the transfer of L3T4+ T cells led to the infiltration of L3T4+ T cells into the recipients' tumors, causing their eradication.

KEYWORDS: eradication of Meth A fibrosarcoma, adoptive immunotherapy, L3T4???lymphocyte, Corynebacterium parvum-Pyridine Extract Residue

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Eradication of immunologically-syngeneic tumors was achieved by adoptive chemotherapy using effector cells induced by *Corynebacterium parvum*-Pyridine Extract Residue (CP-PER). A mixture of 2×10^6 Meth A cells and 0.1 mg CP-PER was subcutaneously inoculated into the back of donor BALB/c mice, with the result that their spleen cells showed an antitumor effect 10 to 13 days after the inoculation. These cells were used as immune cells. Recipient mice were inoculated with 1×10^6 Meth A cells, and 2 days later were administered cyclophosphamide. On the following day, 1×10^8 immune cells were adoptively transferred into the recipient mice. As a result, the tumor began to regress 7 to 12 days after the adoptive transfer. An immuno-histochemical study of the donors' spleens and the recipients' regressing tumors revealed that the ratio of $L3T4^+$ T cells to Lyt-2^+ T cells in the donors' spleens was increased and that the infiltrating cells in the recipients' tumors were mainly composed of $L3T4^+$ T cells. This confirmed that the transfer of $L3T4^+$ T cells led to the infiltration of $L3T4^+$ T cells into the recipients' tumors, causing their eradication.

Key words : **eradication of Meth A fibrosarcoma, adoptive immunotherapy, $L3T4^+$ T lymphocyte, *Corynebacterium parvum*-Pyridine Extract Residue**

Some recent studies (1-3) have shown that the main effector cells for the rejection of allografts or for the eradication of syngeneic tumors are Lyt-1^+2^- cells, which are T cells. L3T4 monoclonal antibody, which specifically identifies helper T cells, has been developed and can be compared with Lyt-2 monoclonal antibody, which identifies cytotoxic T cells. We thus attempted to clarify the mechanism of syngeneic tumor eradication by identification of T cell subsets, using anti-L3T4 and anti-Lyt-2 monoclonal antibodies. A model of adoptive chemoimmunotherapy in mice (2) using *Corynebacterium parvum*-Pyridine Extract Residue (CP-PER) (4, 5) was employed. $L3T4^+$ T cells were mainly found as infiltrated cells in regressing tumors. This result suggested that $L3T4^+$ T cells occupy the most significant position among T cells as effector cells in the syngeneic tumor eradication system.

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Materials and Methods

Mice. Male BALB/c mice, 6 to 10 weeks old, were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Shizuoka Prefecture, Japan).

Tumor. Meth A fibrosarcoma cells were obtained from Chugai Central Research Laboratory (Tokyo, Japan).

Corynebacterium parvum. *Corynebacterium parvum*-Whole Cells (CP-WC) (6-8), CP-Pyridine Extract (CP-PE) and CP-Pyridine Extract Residue (CP-PER) used in these experiments were obtained from Ribi Immunochem Res. Inc. (Hamilton, MT, USA). CP-PE and CP-PER fractions were prepared from freshly grown organisms. CP-PE was verified to be free of cells and cell walls by electron microscopy, and it does not induce hepatomegaly or splenomegaly. Unlike CP-PE, CP-PER retains the ability to cause organomegaly.

Comparison of direct antitumor effect of CP-WC, CP-PE and CP-PER. In order to compare the direct antitumor effects of CP-WC, CP-PE and CP-PER, mice were injected with 2×10^6 Meth A cells admixed with 0.1 mg of CP-WC, CP-PE or CP-PER subcutaneously into the back.

Preparation of immune cells. Immune cells (immunized spleen cells) for the adoptive chemoimmunotherapy were harvested from donor mice on different days after injection of Meth A cells admixed with CP-PER.

Adoptive chemoimmunotherapy (ACIT). Recipient mice were inoculated with 1×10^6 Meth A cells subcutaneously into the back or footpad. Two days later they received 100 mg/kg cyclophosphamide (CY), and on the next day they were infused *i. v.* with 1×10^8 immunized spleen cells through the tail vein. As a control, Meth A alone without any other treatment was used. Also, as another control, mice which received normal spleen cells alone or spleen cells immunized by CP-PER alone or by Meth A cells alone were observed.

Immuno-histochemical staining. An avidin-biotin method of immunoperoxidase staining (Vector Labs., Inc., Burlingame, CA, USA)(9) was used to visualize the biotinylated mouse monoclonal antibodies within frozen sections. The sections were fixed in 100% acetone for 5 min and then incubated with 1% normal rabbit serum for 20 min to block non-specific binding. The monoclonal antibodies (1:100 diluted) were incubated on the sections for 1 h at 37°C. Following addition of avidin-biotin-peroxidase reagent, the reaction was visualized by the addition of diaminobenzidine $NiCl_2$-H_2O_2 (DAB). Sections were counterstained with 1% Meyer's hematoxylin in methanol. The immuno-histochemical staining of the donors' spleens and regressing tumors of recipient mice was observed in order to define the changes in the distribution of spleen cells and the changes in the tumor and the lymphocyte subsets of cells infiltrating the regressing tumors of recipient mice.

Results

Comparison of antitumor activity of components of CP (Fig. 1). CP-WC and CP-PER had a strong antitumor effect, and tumors of mice which received CP-WC and CP-PER began to stop growing 7 days after the mixed injection. The tumors were significantly smaller than those of the control group on day 15 ($p<0.01$). On the other hand, CP-PE had an enhancing effect on tumor growth, and tumors of mice which received CP-PE became larger than those of the control group on day 7 ($p<0.05$). These results were unchanged even after increasing the dose of the components of CP. Even if the dose of CP-PE was increased, the tumors of the CP-PE group did not become smaller than those of the control group. CP-PER had stronger activity than CP-WC, so the following experiments were performed using CP-PER.

Immuno-histochemical findings of immunized spleen cells (Fig. 2). $L3T4^+$ T cells and Lyt-2^+ T cells had the same distribution pattern in spleens obtained from mice that had been injected with Meth A cells alone or an admixture of Meth A cells and CP-PER 10 days before. Compared with the spleens of mice that had been injected with Meth A cells alone 10 days before (A and B

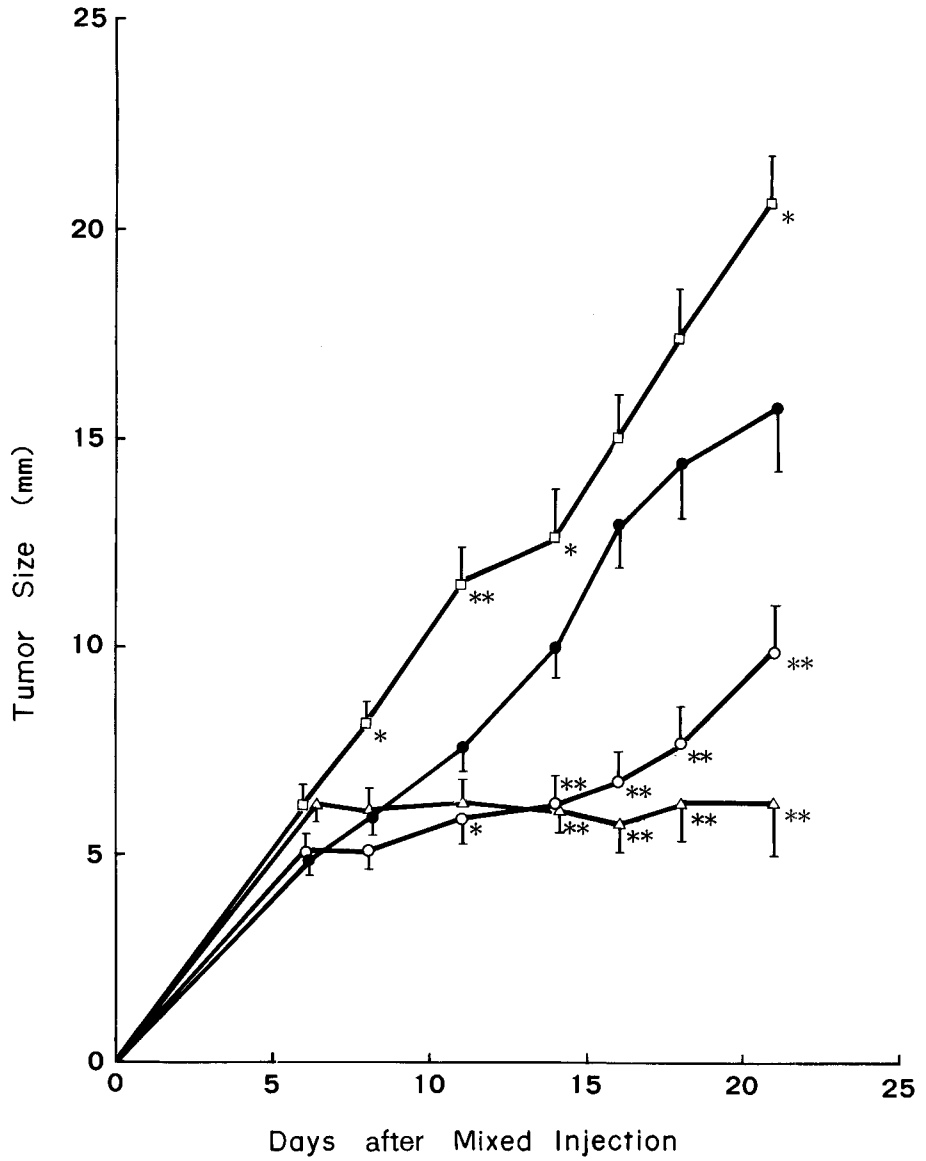


Fig. 1 *Corynebacterium parvum* (CP)-induced tumor regression after subcutaneous injection of an admixture of 2×10^6 Meth A cells and 0.1 mg CP-Whole Cell (○, n=21), CP-Pyridine Extract (□, n=11) or CP-Pyridine Extract Residue (△, n=12) on the back of BALB/c mice. Control mice were injected with 2×10^6 Meth A cells alone (●, n=22). *, $p<0.05$; **, $p<0.01$. Bars, standard errors of means.

in Fig. 2), the number of L3T4$^+$ T cells in the spleens of the mice injected with an admixture of Meth A cells and CP-PER 10 days before (C and D in Fig. 2) increased. However, almost the same number of Lyt-2$^+$ T cells was found in both groups. As a result, the L3T4/Lyt-2 ratio of the spleen cells of mice injected with an admixture of Meth A cells and CP-PER (C/D) became 2 times as much as that of the spleen cells of mice injected with Meth A cells alone (A/B).

Kinetics of complete tumor regression rate

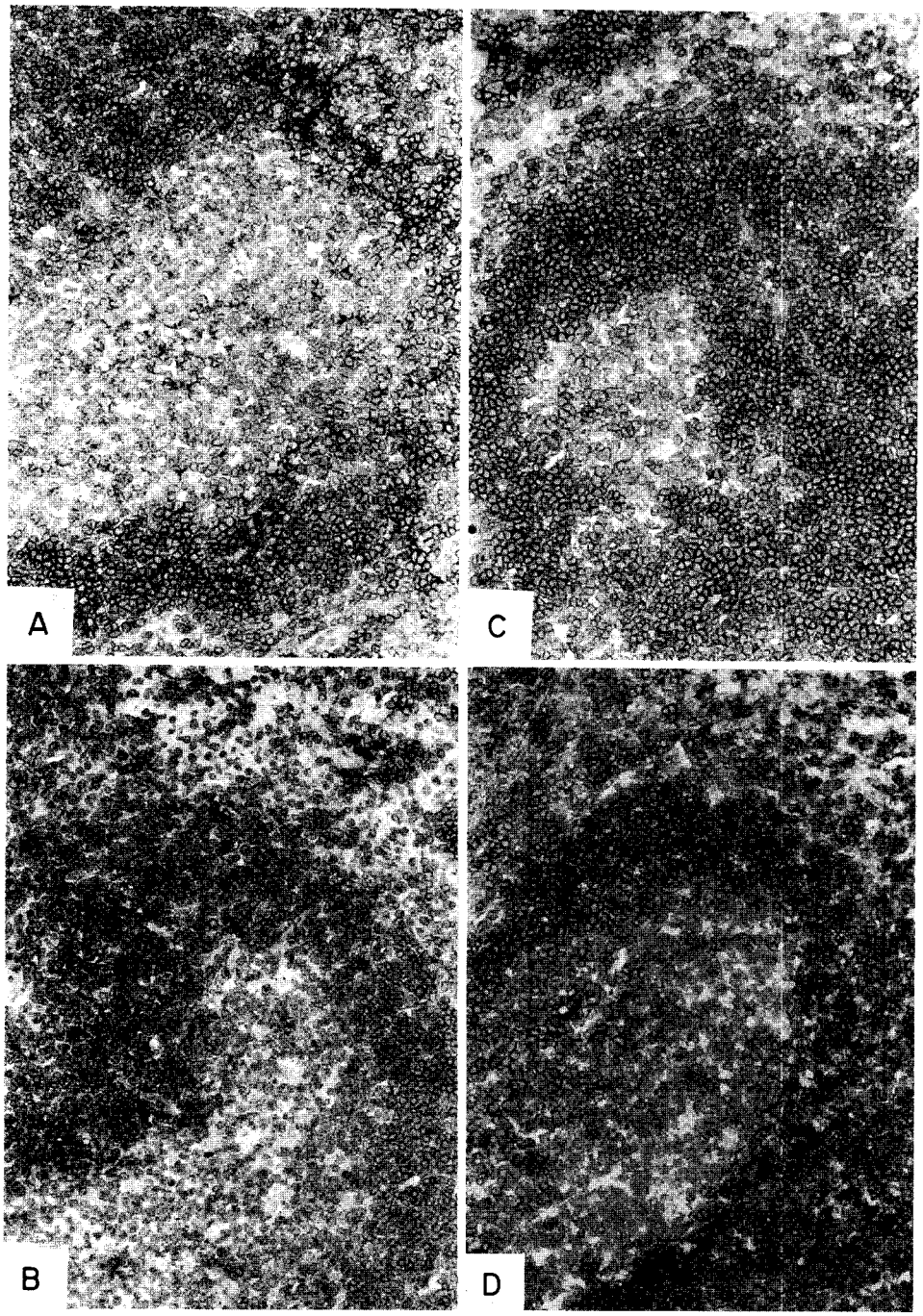
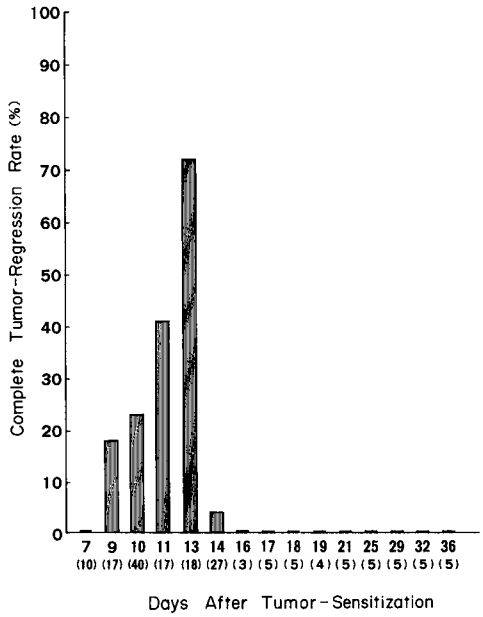


Fig. 2 Immuno-histochemical study of the effect of *Corynebacterium parvum*-Pyridine Extract Residue (CP-PER) administration on the population of lymphocyte subsets in the spleens of immunized donor mice. Donor mice were inoculated with Meth A cells alone (A: ×200, B: ×200) or an admixture of Meth A cells and CP-PER (C: ×200, D: ×200). Ten days after inoculation, a splenectomy was performed, and the spleens were immuno-histochemically stained on continuous sections using monoclonal anti-L3T4 (A, C) or anti-Lyt-2(B, D) antibodies.

Fig. 3 Kinetics of the development and decay of antitumor activity of immunized spleen cells collected from donor mice 7, 9, 10, 11, 13, 14, 16, 17, 18, 19, 21, 25, 29, 32 and 36 days after the mice were injected subcutaneously with an admixture of 2×10^6 Meth A cells and 0.1 mg *Corynebacterium parvum*-Pyridine Extract Residue. Recipient mice were inoculated with 1×10^6 Meth A cells on their footpads or backs subcutaneously, and injected 2 days later with 100 mg/kg cyclophosphamide. On the next day, the mice underwent adoptive transfer of spleen cells. The complete tumor-regression rate was calculated by following formula. Complete tumor regression rate = (No. of tumors completely regressed/n)×100. Recipients' numbers of each group were shown under days numbers.



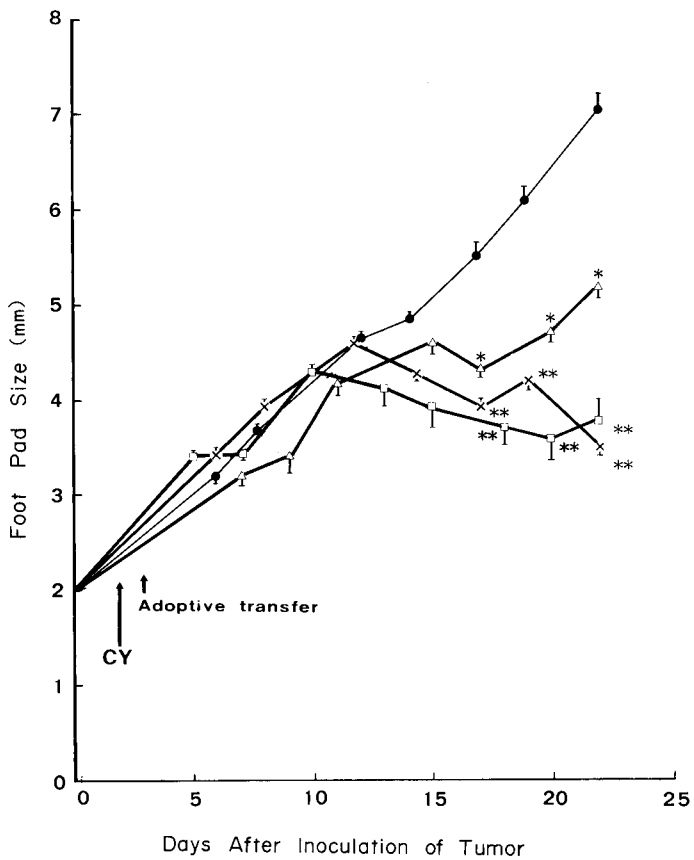


Fig. 4 Growth curve of the tumors of mice which received adoptive chemoimmunotherapy. Recipient tumor-bearing mice were injected on their footpad with 100 mg/kg of cyclophosphamide (CY) and underwent adoptive transfer of 1×10^8 immunized spleen cells collected on day 10 (□, n=4), 11 (△, n=5), 13 (×, n=8). Control mice (●, n=8) were injected with CY and underwent adoptive transfer of normal spleen cells. *, $p<0.05$; **, $p<0.01$. Bars, standard errors of means.

using spleen cells immunized with Meth A cells and CP-PER (*Fig 3*). In the group using spleen cells obtained 7 days after Meth A cells and CP-PER sensitization of the donor, the regressive effect on transplanted tumors was incomoplete. However,

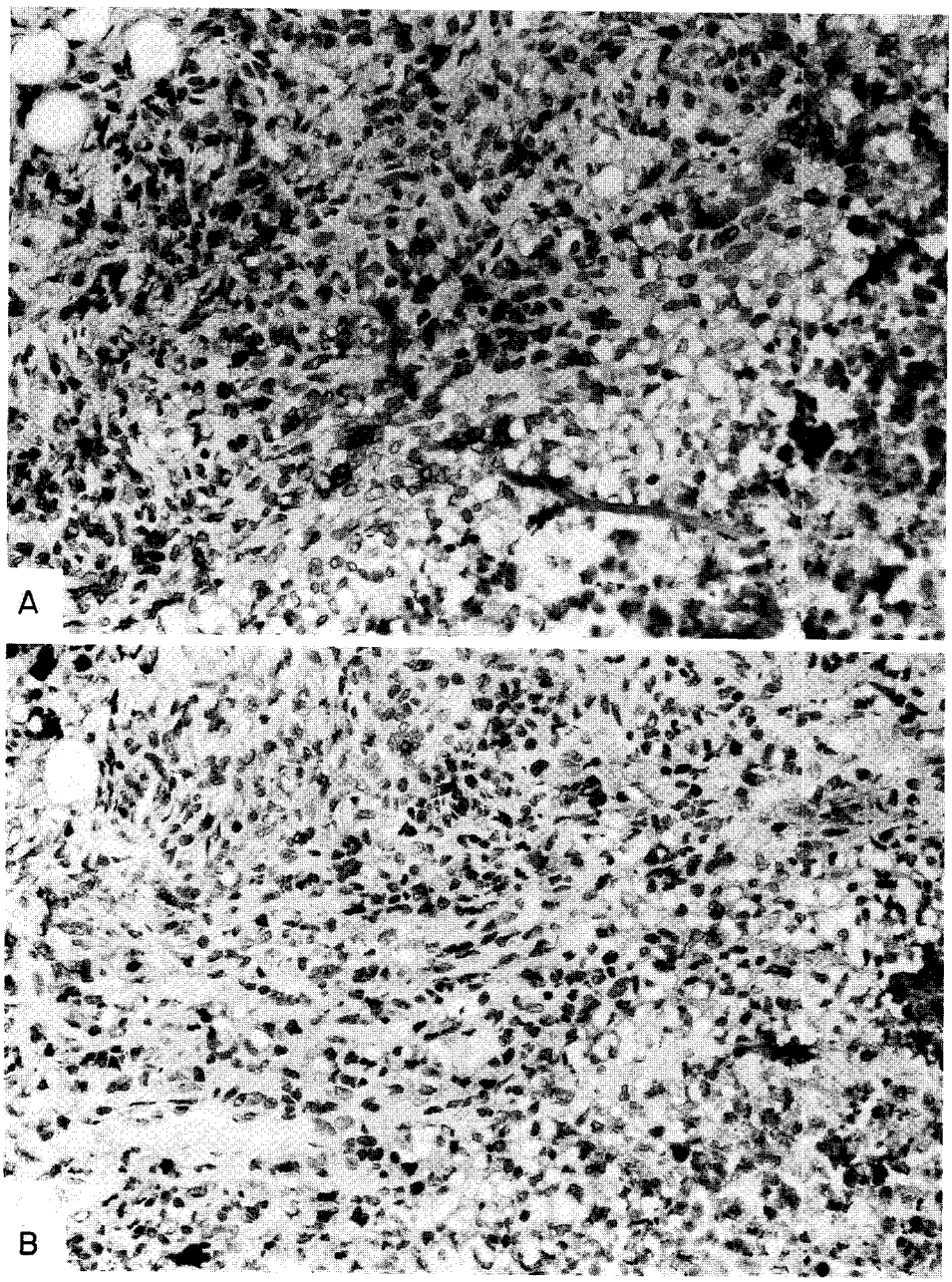


Fig. 5 Immuno-histochemical findings on continuous sections of recipients' tumors. The upper photographs show the outside, and the lower photographs show the inside of the regressing tumor. A: Monoclonal anti-L3T4 antibody was used for this staining. ×200. B: Monoclonal anti-Lyt-2 antibody was used for this staining. ×200.

in the group using spleen cells obtained on the 9th day, complete regression of the recipients' tumors was observed. Although complete tumor-regression was still low in the 9th day group, the rate became higher and higher in the groups receiving spleen cells obtained on the 10th, 11th and 13th days. The complete tumor-regression rate reached a peak (over 70%) in the 13th day group. The rate suddenly dropped to 4% in the 14th day group, and in the groups which received spleen cells obtained 16 days after tumor-sensitization, no complete regressive effect was observed. On the other hand, in the control group of Meth A cells alone and transfer groups of normal spleen cells or spleen cells immunized by CP-PER alone tumor regression was never seen. However, the transfer group of spleen cells immunized by Meth A cells alone showed slight regression of tumors.

Growth curves of the tumors of mice which received ACIT (Fig. 4). Tumors in the foot pads of mice that received adoptive transfer of spleen cells obtained 10, 11 or 13 days after the donor was immunized had an initial period of tumor growth from the day of tumor inoculation to the day of adoptive transfer. After that period, a 7 to 12-day interval of continuous tumor growth was observed between the day of adoptive transfer and the beginning of tumor regression. Tumor regression was not observed soon after adoptive transfer. Seven to 12 days after adoptive transfer, at the time the footpad size was about 5 mm and the tumors were considered to be established, the recipient's tumor began to regress and the footpad became smaller and smaller. This phenomenon was most clearly observed in the 13th day group.

Immuno-histochemical findings of regressing tumors of mice which received ACIT (Fig. 5). Central coagulative necrosis in the recipients' regressing tumors was observed (the lower photographs), and intact tumor cells were observed in the marginal zone of the tumor (the upper photographs). In this zone, only a few mononuclear cells were observed around the normal tumor cells. In the junctional zone between the central necrosis and the marginal zone of intact tumor cells, foamy degenerated tumor cells were observed. Mononuclear cells were observed infiltrating moderately around these foamy degenerated tumor cells. These infiltrating cells were mainly composed of $L3T4^+$ T cells (A), and the number of infiltrating $Lyt\text{-}2^+$ T cells proved to be small (B). Some $L3T4^+$ T cells were observed to be attached to the foamy degenerated tumor cells, and the number of foamy degenerated tumor cells increased as such cells were located nearer to the necrotic area. In addition, only a few macrophages or polynuclear leukocytes, which are found in typical delayed type hypersensitivity (DTH) reactions, were observed in the necrotic area and in the foamy degenerated tumor cell region. When mice were not treated with ACIT, no necrosis or infiltrating mononuclear cells could be observed in the recipients' tumors.

Discussion

Lichtenstein *et al.* (4) have suggested that CP-PER prevents the generation of cytotoxic T cells (CTL) and that CP-PE enhances the activity of suppressor T cells. Berek *et al.* (10) have reported that CP-PER and CP-WC are equal in their antitumor effect, while CP-PE is strongly antitumorigenic. However, in our experiment, CP-PER was highly antitumorigenic, while CP-PE showed little antitumor effect. The differences between these results may be attributed to the different routes of inoculation. In our study, tumor cells mixed with

CP were inoculated subcutaneously, and in the two studies cited, tumor cells alone was inoculated subcutaneously and CP was injected intraperitoneally. We used CP-PER mixed with tumor cells to obtain the most effective augmentation of the concomitant immunity (11, 12) of the tumor-bearing mouse, which was otherwise too weak to be examined by adoptive transfer of spleen cells.

Dye *et al.* (13) have reported that CTL was detected 10 days after the inoculation of CP-WC mixed with P815 mastocytoma cells, leading to complete regression of the tumor with the induction of helper or memory T cells 30 days after the inoculation. No immune cells were detected in our experiment after 16 days. This result is considered to be attributable to differences between the cells induced by CP-WC and the cells induced by CP-PER.

Since 1973, when Leclerc *et al.* (14) pointed out the significance of CTL for the first time, CTL have always been trusted as the main effector cells in the immunological tumor rejection system. However, recent studies (15) have shown that cloned CTL demonstrate a strong effect by Winn assay and systemic transfer, while they have a weak effect *in vivo* if used alone. Lyt-1^+2^- T cells may be more important than CTL as effector cells for tumor rejection in experimental systems using an adoptive immunotherapy model (3, 16-20). In our present experiment using an adoptive chemoimmunotherapy model in mice both immunized spleen cells transferred to the recipient and target cells which infiltrated into the tumor seemed to be mainly $L3T4^+$ T cells. This result conflicts with the conventional interpretation of CTL as being the main effector cells for tumor rejection, while it supports reports which have suggested that transferred $L3T4^+$ T cells, namely helper T cells, induce a DTH reaction (3, 17-19, 21), and that DTH precursor cells are transferred and matures into DTH effector cells. The idea that DTH precursor cells (22) matures into DTH effector cells is also supported by a report (23) which has suggested that the requirements for adoptive immunity are not the derivation of effector cells from the host, but the growth of such transferred cells in the host, although at the present time this is no more than conjecture.

Greenberg *et al.* (2) showed that CY has a direct tumoricidal effect, but this effect could not be observed in our experiment. They also observed that CY had potentially facilitating effects on host tumor immunity, and that therapy with immune cells without CY had no apparent *in vivo* antitumor effect. We observed the same effect. Nakajima *et al.* (24) also showed that suppressor T cells involved in the DTH reaction were eliminated after CY treatment.

In our experiment shown in Fig. 3, the 13 day group showed over 70% of complete tumor regression rate. However in Fig. 4, the tumor growth curve of the 13 day group did not become back to 2 mm level. This discrepancy is considered to be due to the difference of calculation, that is, the difference between the percentage of regression cases in Fig. 3 and the average size of tumors including growing tumors in Fig. 4.

$L3T4^+$ T cells were found to be present near tumor cells subjected to foamy degeneration rather than on the margin of the tumor, while very few of Lyt-2^+ T cells were observed. Our study also revealed no nonspecific inflammation involving infiltration of macrophages or polynuclear leukocytes such as are found in typical DTH reactions induced by DTH effector cells. These findings give the impression that $L3T4^+$ T cells directly attack tumor cells, suggesting the possibility of the direct action of DTH effector cells on tumor cells, as well as the action of $L3T4^+$ CTL (25) on tumor cells. There are few other reports similar to ours

which describe experiments on immunohistochemical identification of effector cells of syngeneic tumor eradication using an immunotherapy model. Reichert *et al.* (7) have reported that cells infiltrated in a tumor contain seven times more Lyt-1^+ T cells than Lyt-2^+ T cells, and Uede *et al.* (26) have found that Lyt-1^+2^+ T cells infiltrated a rejected tumor. There is also a report (27) indicating that the tumor determines the Lyt phenotypes of effector cells.

We believe that the immunological analysis of tumor regression by immunotherapy using a mouse model as described in this paper will contribute to its future clinical application.

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