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## The role of CD4<sup>+</sup> and CD8<sup>+</sup> cell subsets in the growth-control of MCH-13 fibrosarcoma

KSENIJA LUČIN<sup>1</sup>, FILIP ČULO<sup>2</sup> and NIVES JONJIĆ<sup>1</sup>

<sup>1</sup> Department of Pathology and Pathological Anatomy, Medical Faculty, University of Rijeka, Olge Ban 22, 51000 Rijeka, Croatia

<sup>2</sup> Department of Physiology, Medical Faculty, University of Zagreb, Šalata 3, 41000 Zagreb, Croatia

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**SUMMARY.** – The aim of this study was to analyse the cells involved in immunity to immunogenic MCH-13 fibrosarcoma. The role of T lymphocyte subsets was investigated by depletion of these cells by *in vivo* administration of anti-CD4 and anti-CD8 monoclonal antibodies. The effect of T cell subsets depletion on the growth of primary tumor graft as well as the ability of tumor-primed mice to reject the secondary tumor challenge was tested. In the initial phase of the primary tumor both T cell subsets are required for the control of the tumor growth. In contrast, in the later phase, only CD8<sup>+</sup> cells are essential. Postexcision immunity was tested on the ability of mice to reject a secondary tumor graft of the same tumor. Primed, nondepleted mice were resistant to the secondary tumor graft. Depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes abolished the resistance. However, this was pronounced only in anti-CD8 treated mice, while anti-CD4 treatment had only a partial effect. In an attempt to characterise tumor infiltrating lymphocytes the immunohistological analysis of primary tumor tissue was performed which confirmed the role of both T lymphocyte subsets.

### INTRODUCTION

Cell-mediated immunity against tumors has been the subject of intense research interest for many years. The role of the immune system in the control of tumor growth has been investigated on various experimental tumors. The potential of experimental tumors to induce an immune response is variable depending on the nature of carcinogen and the presence of tumor-specific antigens on the surface of tumor cells. Some virus-induced tumors (16) and tumors induced by UV light (26) can stimulate a strong immune response. These tumors may fail to grow after transplantation to immunocompetent syngeneic recipients or may undergo regression after a certain period of growth. Chemically induced tumors are generally less immunogenic and require some form of immunization for inducing a rejection response (4, 19). Mch-13 is an experimental tumor induced by methylcholanthrene, a chemical carcinogen. It grows progressively in

the unimmunized host, but after a certain form of immunization, for example surgical removal, the tumor can be completely rejected (4). It is well known that the immune response to primary and secondary tumor graft is mediated by the T lymphocytes (3, 9, 19, 20, 22), but the effector mechanisms responsible for the tumor growth control and rejection are still poorly understood. In an attempt to ascertain the role of different T lymphocyte subsets in tumor rejection we used the method of *in vivo* depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. Our results indicate that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells play an important role in the immune response to primary tumor and secondary tumor graft. While CD4<sup>+</sup> T cells are involved in the initial phase of immune response to primary tumor, CD8<sup>+</sup> cells are required for the second, effector phase. The immunity to secondary tumor challenge is also mediated by both T lymphocyte subsets, but the role of CD8<sup>+</sup> cells appears to be predominant.

## MATERIAL AND METHODS

### Experimental animals

Female CBA mice 10–12 weeks old were used in the experiments. They were fed with standard diet and water *ad libitum*.

### Tumor

MCH-13 fibrosarcoma syngeneic in CBA mice was induced by injecting subcutaneously adult CBA mice with 0.5 mg 20-methylcholanthrene (Fluka A. G., Buchs S. G., Switzerland). The tumor was maintained *in vivo* and the 79th passage of the tumor was used. In all experiments tumor was initiated by injecting  $10^5$  tumor cells in a volume of 50  $\mu$ l of PBS intradermally in the dorsal region. The growth was monitored by measuring changes against time in the mean of two perpendicular tumor diameters.

### Depletion of T-cell subsets

For *in vivo* depletion of T cell subsets mAbs 169.4 (anti-CD8) and 191.1 (anti-CD4) were used (6). Hybridomas were grown in the peritoneal cavity of (LOU $\times$ DA)<sub>F1</sub> rats and ascitic fluid, purified by double ammonium-sulfate precipitation, was used. After dialysis against PBS the stocks were sterilely filtered and stored frozen. Concentration of rat immunoglobulins was determined by radial immunodiffusion (RID plates, Serotec). MABs were injected intraperitoneally (1 mg/dose) in the volume of 1 ml of PBS. Control animals received the same volume of PBS. Depletion was further maintained by repeated injections of mAbs after 7 and 14 days.

### Monitoring of T-cell subset depletion

The two-color immunofluorescent method was used for the monitoring of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subset depletion after *in vivo* treatment with anti-CD4 and anti-CD8 monoclonal antibodies, respectively. Spleens were dissociated mechanically and cell suspension was filtered through nylon mesh. Erythrocytes were lysed with buffered ammonium chloride solution. The cells were incubated with FITC-conjugated mAbs to CD8 molecule and phycoerythrin (PE)-conjugated mAb to CD4 molecule (both purchased from Becton Dickinson, Mountain View, CA, USA). Dead cells were stained with propidium iodide (Serva, 1  $\mu$ g/ml) and excluded during acquisition of 20000 viable cells using the FACScan flow cytometer (Becton Dickinson). Two-color immunofluorescent data were displayed as contour graphs, and log intensities of green fluorescence (FITC) plotted on the x-axis and log intensities of red fluorescence (PE) plotted on the y-axis using consort 30 software (Becton Dickinson).

### Immunohistological staining

Immunohistological analysis of tumor tissue was performed by the ABC method on the cryostat sections as described (14). After overnight drying at room temperature, sections were fixed in acetone for 7 min and then incubated with 1% normal horse serum for 30 min to block nonspecific binding. The mAbs that recognize CD4 (undiluted supernatant of GK 1.5 hybridoma) and CD8 (ascites of 169.4 hybridoma, 1:2000) molecules were incubated on the sections for 30 minutes at room temperature. Following the addition of secondary, biotinylated antibodies (Biotin-SP-mouse anti-rat IgG, Dianova), endogenous peroxidase activity was blocked by incubation in H<sub>2</sub>O<sub>2</sub> (0.3% in methanol). Slides were then incubated with avidin-peroxidase solution (1:5000) for a further 30 min (Horseradish peroxidase-avidin D, Vector). The antibody binding was visualized by the addition of diaminobenzidine (NiCl<sub>2</sub>-H<sub>2</sub>O<sub>2</sub>-DAB) (3,3'-Diaminobenzidine tetrahydrochloride, Sigma). Slides were then mounted with glycerin-gelatine and investigated under light microscope.

### Statistical analysis

Statistical analysis was carried out by Student's *t* test.

## RESULTS

### Effect of T cell subset depletion on the primary tumor growth

#### Depletion on the day of immunization

Mice were *i.d.* immunized with  $1 \times 10^5$  Mch-13 cells. On the day of immunization the mice were depleted of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes by *in vivo* treatment with anti-CD4 anti-CD8 mAbs, respectively. This treatment produced a dramatic reduction of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively (Figure 1). Using direct immunofluorescence, less than 1% of CD4<sup>+</sup> and CD8<sup>+</sup> cells in the spleen of mAb-treated mice were found as early as 24 h after the first mAb injection. Depletion of both T-cell subsets was performed by simultaneous treatment with a combination of anti-CD4 and anti-CD8 mAbs. Depletion was repeated after 7 and 14 days.

The effect of T cell depletion on the tumor growth is shown in Figure 2a. Control, nondepleted animals developed a tumor that grew slower than tumors in T cell-depleted animals, especially double-depleted. After the 15th day of immunization the difference in the growth rate between depleted and control groups was statistically significant ( $p < 0.01$ ). The data indicate that both T cell subsets are involved in the initial phase of immune response to the tumor growth.

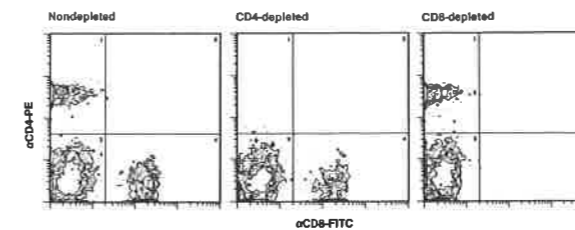


FIGURE 1. Cytofluorometric analysis of T-cell subsets in CBA mice after treatment with anti-CD4 and anti-CD8 mAbs *in vivo*. Fifteen days after the first injection of monoclonal antibodies spleen cells were double-stained by direct immunofluorescence using phycoerythrin (PE)-conjugated mAb to CD4 molecule and FITC-conjugated mAb to CD8 molecule.

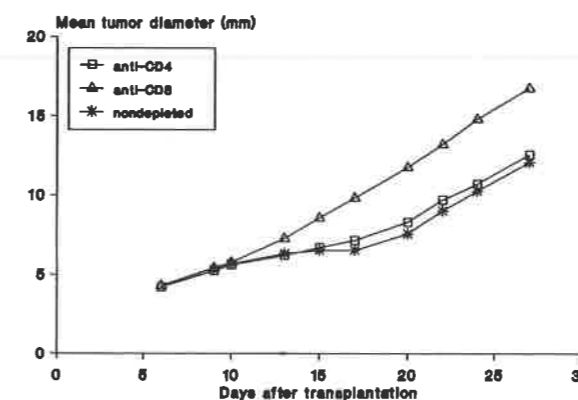
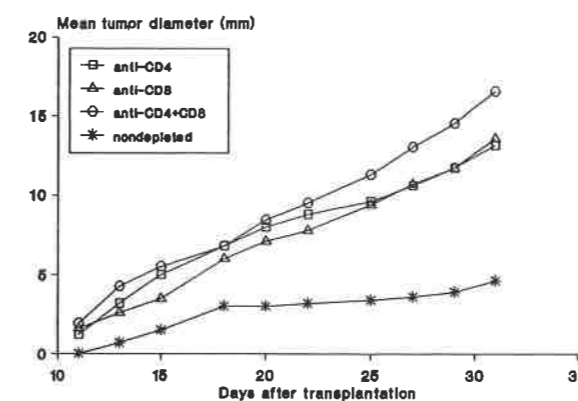


FIGURE 2. Effect of *in vivo* depletion of T lymphocyte subsets on the growth of syngeneic tumor graft. CBA mice were injected *i.d.* with  $1 \times 10^5$  MCH-13 tumor cells. Groups of mice were treated with rat anti-CD4 and anti-CD8 mAbs in order to deplete CD4-subset, CD8 subset and both subsets of T lymphocytes, respectively. (Means of 6 mice per group) a) mAb treatment started on the day of tumor transplantation; b) mAb treatment started on day 9 after tumor transplantation

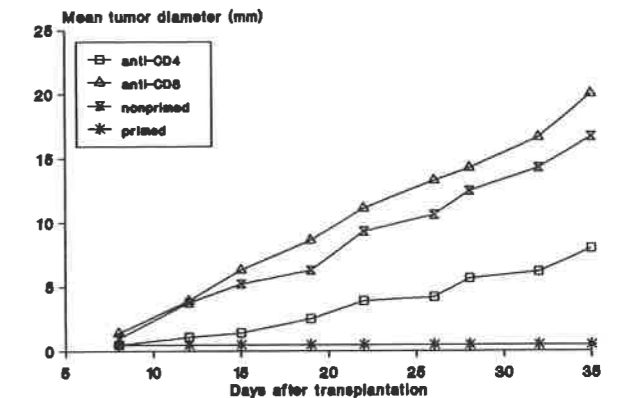


FIGURE 3. Effect of T cell subsets depletion on the resistance to secondary tumor challenge. Two weeks after the excision of primary tumor graft mice were treated with a challenge dose of the same tumor and additional treatment with anti-CD4 or anti-CD8 mAbs was performed (Means of 6 mice per group).

### Depletion on day 9 after immunization

In order to assess the role of T cell subsets in the effector phase of immune response, mice were treated with monoclonal antibodies 9 days after immunization (Figure 2b). Depletion was repeated on the 7th and 14th day after the first injection. Control animals, as well as those treated with anti-CD4 mAbs developed a tumor of similar size, whereas in anti-CD8 treated animals the tumor grew more rapidly and the difference was statistically significant ( $p < 0.05$ ). Depletion of CD8<sup>+</sup> cells, which resulted in faster tumor growth, indicates that these cells are the main effector cells in the immune response to primary tumor growth.

### Effect of T-cell subset depletion on secondary tumor growth

Mice were immunized with  $1 \times 10^5$  Mch-13 cells *i.d.* The tumor was excised on day 9 after grafting. Post-excision immunity was tested as the ability of mice to reject a secondary graft of the same tumor, given two weeks after excision. In order to discriminate the role of T lymphocyte subsets in post-excision immunity, the mice were treated with anti-CD4 and anti-CD8 monoclonal antibodies on the day of secondary tumor graft. At this time the same number of tumor cells was given to control, nonprimed mice. Depletion was repeated after 7 and 14 days. The results are shown in Figure 3. All nondepleted animals were resistant to secondary tumor graft. Depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes abolished the resistance. Yet, immune, CD4<sup>+</sup>8<sup>+</sup> animals showed marked enhancement of tumor growth, while anti-CD4 treatment had only a partial effect.

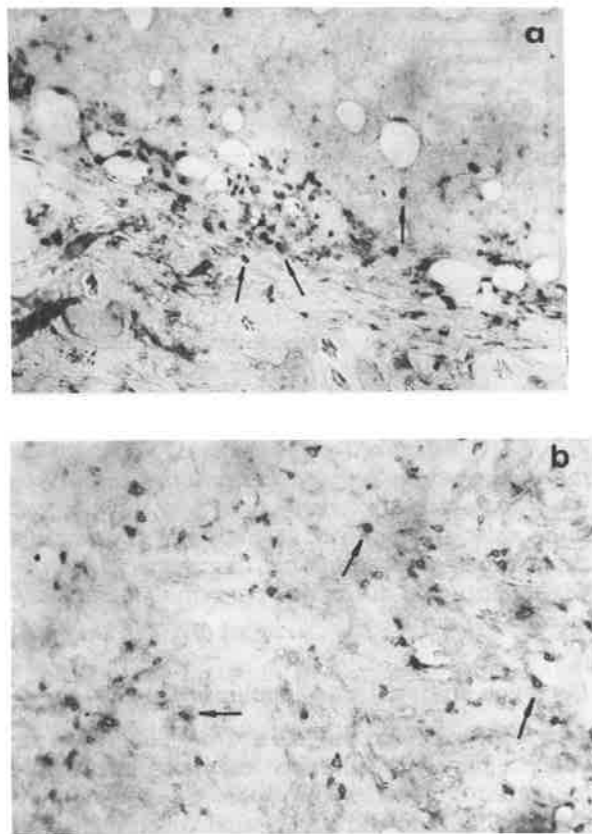


FIGURE 4. Primary tumor tissue of control, non-depleted animal stained with anti-CD4 and anti-CD8 mAbs. CD4<sup>+</sup> cells in the form of dense aggregates are present in tumor periphery and penetrate to the deeper part where they are rare (a). CD8<sup>+</sup> T cells in the central part of the tumor (b). Magnification  $\times 125$ .

There was a statistically significant difference in the tumor diameter between anti-CD4 and anti-CD8 treated mice as well as between anti-CD4 treated and nonprimed ( $p < 0.05$ ), but not between anti-CD8 and nonprimed.

#### Immunohistological analysis of the primary tumor tissue

In order to ascertain the phenotype of cells that infiltrate primary tumor tissue we performed immunohistological analysis of MCH-13 fibrosarcoma of immunocompetent as well as anti-CD4 and anti-CD8 treated animals. Immunohistology was done on tumors of two mice per particular group. The infiltrate of primary tumors of control animals was

composed of both T lymphocyte subpopulations. Infiltration of CD4<sup>+</sup> T cells preceded the infiltration of cells of the CD8<sup>+</sup> phenotype. With progression of tumor growth a relative decrease in the number of CD4<sup>+</sup> cells and a relative increase in the number of CD8<sup>+</sup> cells was observed. Two weeks after immunization, CD4<sup>+</sup> T cells were present mainly on the periphery of the tumor (Figure 4a), penetrating to the deeper parts, where they were rare and scattered. In the central part of the tumor there was a great prevalence of CD8<sup>+</sup> T cells over CD4<sup>+</sup> (Figure 4b), but depletion of CD8<sup>+</sup> T cells was followed by a considerable increase in the number of CD4<sup>+</sup> T cells. On the contrary, in the group of anti-CD4-depleted animals, there were only a few CD8<sup>+</sup> T cells.

#### DISCUSSION

It is well known that T lymphocytes play an important role in the immune response to tumor cells, but the effector mechanisms responsible for immunological control and rejection of experimental tumors are still poorly understood. The role of T lymphocyte subpopulations in anti-tumor immune response appears to vary depending on the tumor system examined. The role of CD8<sup>+</sup> effector cells is confirmed in the case of mouse Sa-1 (21), Meth-A fibrosarcoma (4, 19), P-815 mastocytoma (7, 17, 18) and L5178Y lymphoma (1) leading to the complete regression of tumor in tumor-bearing recipients. In contrast, for some experimental models CD4<sup>+</sup> (or Lyt-1<sup>+</sup>) cells have been shown to be the predominant effector cells (8, 10, 12, 13). Some studies have demonstrated that these noncytolytic T cells exert their anti-tumor effect without the participation of CTL (10, 13). The explanation is that different types of tumor antigen stimulate predominantly one particular type of antitumor T-cell subset. The question is how CD4<sup>+</sup> T cells can exert their antitumor activity. Cytolytic activity mediated by lymphotoxin or TNF is possible (15), although Fujiwara et al. (11), have demonstrated in the model of X5563 plasmocytoma that CD4<sup>+</sup> T cells cannot exert direct cytolytic activity on tumor cells. Another possibility is the activation of nonspecific effector mechanisms. In this case, the action of CD4<sup>+</sup> T cells depends on the presence of adherent cells (11, 33). However, CD4<sup>+</sup> suppressor cells, capable of limiting the effectiveness of the immune response to immunogenic tumors were found in some tumor systems (1, 2, 23, 24).

In our tumor model T cell immunity to MCH-13 fibrosarcoma is mediated by both lymphocyte subpopulations. Depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes on the day of immunization caused an abrogation of antitumor immune response that resulted in enhancement of tumor growth. This implies that in the initial phase of tumor growth both T lymphocyte subpopulations are important in im-

munological control of the primary tumor growth. In the subsequent phase, which can be designated as an «effector phase», only CD8<sup>+</sup> T cells are involved, since only the depletion of CD8<sup>+</sup>, but not of CD4<sup>+</sup> T cells, affects the tumor growth. The role of CD4<sup>+</sup> T cells in the initial phase is probably to provide IL-2, a helper factor for proliferation and differentiation of CD8<sup>+</sup> effector cells. Much data obtained in experiments with the adoptive transfer of specific sensitized T lymphocytes support the view on cooperation between CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes and the role of CD4<sup>+</sup> cells as helper cells (5, 27, 30, 31).

The ability of mice with excised tumors to reject second tumor implant is usually considered proof of antitumor immune response and a consequence of the presence of immunogenic tumor-associated transplantation antigens (TATA) on tumor cells (25, 28). MCH-13 tumor appears to be an immunogenic tumor, since all mice that were excised on day 9 after priming rejected the inoculum of the same tumor cells given two weeks later. Depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes abolished the resistance. This effect was not as pronounced in the anti-CD4 treated mice as it was in the group of anti-CD8 treated mice, since tumors in CD4-depleted mice grew slower and in CD8-depleted mice faster than in non-primed controls. In the case of Meth-A fibrosarcoma the postexcision immunity that existed two weeks after excision of primary Meth-A tumor was functionally eliminated by either anti-Ly-1 or anti-Ly-2 monoclonal antibody and complement (4). Thus, the expression of this immunity depended either on Ly-1<sup>+</sup>2<sup>+</sup> T cells, or on two separate T-cell populations, Ly-1<sup>+</sup>2<sup>-</sup> and Ly-1<sup>-</sup>2<sup>+</sup>. However, this immunity was not expressed immediately, but the tumor grew for about 8 days before being rejected. It was explained as the period required for the memory T cells to give rise to the secondary production of effector T cells. Our results were somewhat different, as tumor-excised mice rejected second tumor challenge immediately and completely. The main effector cells that mediated the rejection response in our experiments were probably CD8<sup>+</sup> T cells, while the role of CD4<sup>+</sup> T cells is not completely clear.

It is well known that the expression of CD4 and CD8 molecules is not restricted to particular lymphocyte subpopulations, but can also be found on some cells of the monocyte-macrophage line and NK cells, respectively. In any case, MCH-13 fibrosarcoma appeared to be resistant to lysis mediated by NK cells (unpublished data). Taken together with the fact that immunocompetent, primed mice are able to reject the secondary tumor inoculum, in contrary to non primed mice, we believe that T lymphocytes can be considered as potential mediators of immune response to MCH-13 fibrosarcoma.

Immunohistological characterization of tumor-infiltrating lymphocytes revealed the presence of both T lymphocyte subsets in the primary tumor tissue. The finding that infiltration of CD4<sup>+</sup> T cells precedes the appearance of CD8<sup>+</sup> T cells is in accordance with the results obtained with rat T-9 gliosarcoma (32). The accumulation of CD8<sup>+</sup> T cells is regulated by the lymphocyte migration factor, chemotactic factor produced by CD4<sup>+</sup> T cells (29). This could explain the decreased number of CD8<sup>+</sup> T cells in the tumor tissue of anti-CD4-treated animals.

Our results illustrate the significance of both T lymphocyte subsets for immunological control of MCH-13 fibrosarcoma. The main effector cells are probably CD8<sup>+</sup> T cells, but many questions, especially those concerning the precise role of CD4<sup>+</sup> T cells, still remain open.

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