Jpn. J. Cancer Res. 80, 895–903, September 1989

Combination Therapy of Colon Carcinoma 26 in Mice with Recombinant Human Interleukin-2 and Interferon-α A/D: Occurrence of Large Granular Cells in the Tumor

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The antitumor effects of recombinant human interleukin-2 (rIL-2), in combination with recombinant human interferon-α A/D hybrid (rIFN-α A/D) on colon carcinoma 26 (colon 26) in mice were examined histologically. Colon 26 was transplanted subcutaneously into female BALB/c mice on day 0. The mice bearing the tumor received intramuscular injection of rIL-2, rIFN-α A/D or the combination of rIL-2 and rIFN-α A/D for 2–10 consecutive days starting on day 7. Mice were killed on days 9, 13, 17 and 21. After day 13, growth of the tumor was significantly suppressed in the mice treated with rIL-2 or rIFN-α A/D alone and was stopped in the mice treated with rIL-2 in combination with rIFN-α A/D. Histologically, tumor necrosis developed in all treated groups, though the degree was the most severe in the group receiving combination treatment. Many large cells (about 15–30 μm in diameter) infiltrated into the tumor, and they had Thy-1 surface antigen and many periodic acid-Schiff-positive round granules in the cytoplasm. The incidence of these large granular cells was correlated well with the reduction in tumor weight. The ultrastructural features of the large granular cells were very similar to those of murine large granular lymphocyte-like cells maintained *in vitro* in an IL-2-containing medium. The present large granular cells appear to be a kind of activated lymphoid cells.

Key words: Interleukin-2 — Interferon-α A/D — Colon carcinoma 26 — Combination therapy — Large granular cell

Recent progress in recombinant DNA technology has made it possible to produce purified recombinant lymphokines in large amounts, and some of them have been applied to the treatment of patients suffering from cancer or viral diseases. It has been demonstrated that recombinant human interleukin-2 (rIL-2) and recombinant human interferon α A/D (rIFN-α A/D), a hybrid of rIFN-α A and rIFN-α D, have potent antitumor effects on murine tumors *in vivo*. Recently, the combination treatment with rIL-2 and rIFN-α A/D was proved to have beneficial antitumor effects on murine tumors *in vivo*.[1-3] In our previous study, [1] systemic administration of rIL-2 in combination with rIFN-α A/D markedly suppressed the growth of colon 26 and Meth-A fibrosarcoma in mice. The mechanisms of the antitumor effects of rIL-2 in combination with rIFN-α A/D are not fully understood. It has been demonstrated that rIFN-α A/D has a direct antiproliferative effect on murine cells *in vitro*[4,5] and host-mediated immunomodulatory effects, including augmentation of natural killer (NK) cell activity,[6,7] activation of macrophages[8,9] and activation of T cell immunity[10] in mice. On the other hand, rIL-2 has no direct antiproliferative effects, but it has various host-mediated immunomodulatory effects including the induction of lymphokine activated killer (LAK) cell activity[11,12] and augmentation of NK cell activity[6,11] and cytotoxic T lymphocyte (CTL) activity[11] in mice. Therefore, in the case of systemic administration of rIL-2 in combination with rIFN-α A/D, host-mediated immunomodulatory effects seem to be more important than the direct effects on the tumor cells.

In the present study, we investigated histopathologically the subcutaneously transplanted colon 26 solid tumors in mice which received rIL-2 alone, rIFN-α A/D alone, or the combination of rIL-2 and rIFN-α A/D to elucidate whether or not cell-mediated antitumor action occurs in the tumor tissues.

MATERIALS AND METHODS

Drugs rIL-2 was provided by Applied Microbiology Laboratories of our Division (TGP-3, Lot.609). Specific activity of the rIL-2 corresponds to 1.17×10^7 JRU/mg (JRU: Japan Reference Unit, established by NIH of Japan in April 1989). The purification procedure, and biochemical and biological properties of the rIL-2 have been described in detail elsewhere.[13-17] The rIFN-α A/D was kindly provided by Nippon Roche Inc. (Kanagawa). The specific activity was 1.5×10^8 IU/mg protein when titrated on an MDBK-VSV system[18] using the WHO standard, Gxa 01-901-535. Both rIL-2 and rIFN-α A/D

were dissolved in saline containing 5% normal mouse serum.

Animals Female BALB/cAnNCrj (BALB/c) mice were purchased from Charles River Japan Inc. (Kanagawa) and used for the experiments at 9 weeks of age. The mice were kept under specific pathogen-free conditions in our laboratory, fed a chow diet (CE-2; CLEA Japan, Inc., Osaka) and given water *ad libitum*.

Tumor Colon carcinoma 26 (colon 26) was obtained from the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo) in 1983 and has been maintained in BALB/c mice by subcutaneous passage in solid form. A tumor homogenate was made and adjusted to a 20% (w/v) solution with Hanks' solution, then 0.1 ml of the homogenate was transplanted into the subcutaneous tissue of the left abdominal skin of mice on day 0. Forty-eight mice with established tumor were obtained, divided into 4 groups on day 7 and used for the experiment. The tumor diameter was measured with a caliper, and the size of each tumor was expressed as the mean value of the maximal longitudinal diameter and the maximal transverse diameter.

Drug injection The 12 mice in each group received an intramuscular injection of vehicle (5% normal mouse serum in saline, vehicle group), 10 μg of rIL-2 (rIL-2 group), 2×10^5 IU of rIFN-α A/D (rIFN-α A/D group), or the combination of 10 μg of rIL-2 and 2×10^5 IU of rIFN α A/D (combination group) daily from day 7 to day 16. The volume of solution injected was adjusted to 0.1 ml/mouse in all treatments. Our previous studies had shown that the above doses of rIL-2 alone, rIFN-α A/D alone and their combination were sufficient to suppress the growth of subcutaneously established colon 26.[1]

Histological examination of the tumor Three mice from each group were autopsied on days 9, 13, 17 and 21. The tumors were removed, weighed and fixed in 10% buffered formalin (pH 7.4). A tissue block was obtained from a whole-cut section through the largest longitudinal dimension of the tumor mass and was embedded in Tissue Prep (Fisher Scientific, USA). Two thin sections were made from all mice and stained with hematoxylin and eosin (HE) or periodic acid-Schiff and hematoxylin (PAS) and were examined with a light microscope.

Incidence of large granular cells in tumor tissue was measured in the mice killed on day 13, because tumor weight was markedly reduced after day 13 in the combination group. Total area of a tumor tissue section of each mouse was measured with an image analyzer system (IBAS 2000, Zeiss, West Germany), and the total number of large granular cells in each tumor section stained with PAS was counted under a light microscope. The values were given as the total number of cells/unit area of tumor tissue. The correlation between tumor weight and incidence of large granular cells was examined.

Immunohistochemical examination of the tumor The tumors in the mice of the combination group (killed on days 13 and 17) were removed and cut into two pieces through the largest longitudinal dimension of the tumor mass. One piece was fixed in 10% buffered formalin for the above histological examination. The other half was cut into 3 pieces. Two of the 3 pieces were used for immunohistochemical examinations, and the remaining piece was prepared for ultrastructural examination. For immunohistochemical examination, one piece was sliced into sections (2 mm thick), fixed in 95% ethanol at 4°C overnight, and embedded in Tissue Prep according to the method of Saint-Marie.[19] Following this, thin sections were made.

The other piece was frozen in isopentane which had been precooled in an acetone and dry ice mixture. The frozen tumor tissue was sectioned using a cryostat and stored in a freezer at −30°C until immunohistochemical examinations. The cryostat sections were air-dried and fixed in acetone at 4°C for 30 min before immunostaining.

To examine the cell surface antigens of the lymphoid cells in the tumor tissue, fluorescein isothiocyanate (FITC)-conjugated anti-Thy-1.2 monoclonal antibody (ICN Immuno Biologicals, USA), FITC-conjugated anti-Lyt-1 and anti-Lyt-2 monoclonal antibodies (Becton Dickinson, USA), anti-Thy-1 monoclonal antibody (YBM/29/2.1, Sera-lab, UK) and anti-mouse IgG monoclonal antibody (YA2/40-H(LK), Sera-lab) were used. The deparaffinized sections and cryostat sections were incubated with the above FITC-conjugated antibodies (1:100) and were examined under a fluorescence microscope (Nikon, Japan). The sections incubated with YBM/29/2.1 monoclonal antibody (rat IgG, 1:1000) or YA2/40H(LK) monoclonal antibody (rat IgG, 1:1000) were stained using a Vectastain ABC-AP Kit for rat IgG (Vector Laboratories, Inc., USA). These sections were counterstained with hematoxylin and were examined under a light microscope.

Ultrastructural examination of the tumor Pieces of the tumor tissue from mice of the combination group (killed on day 13) were cut into small blocks and fixed in 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH. 7.4), postfixed in 1% osmium tetroxide in the same buffer, and embedded in epoxy resin (Epok 812). Ultrathin sections were made, doubly stained with uranyl acetate and lead citrate, and observed under a JEM-1200EX electron microscope (Japan Electron Optics Lab., Tokyo).

RESULTS

Antitumor effect The tumor sizes in mice from day 7 to day 21 are shown in Fig. 1. Tumor growth was slightly suppressed in the mice given rIL-2 (10 μg/day) or rIFN-α A/D (2×10^5 IU/day) alone as compared to the

vehicle group. The tumors in mice given the combination treatment showed a marked reduction in size after day 13. As shown in Table I, administration of either rIL-2 or rIFN-α A/D alone moderately suppressed the increase in tumor weight. The suppressive effect was somewhat more prominent in the rIFN-α A/D group than in the rIL-2 group. In the combination group, increase in tumor weight was markedly suppressed, and there was no increase after day 13.

Histology of the tumor There was slight focal necrosis of the tumor tissue on day 9 in all groups. The tumor necrosis progressed slightly with advancing time in the vehicle group (Table II). Administration of rIL-2 or rIFN-α A/D alone accelerated the development of focal tumor necrosis. In mice given the combination treatment, all tumors were found to be extensively necrotic on day 17 and day 21 (Fig. 2.). In and around the tumor tissue, there was marked infiltration of large cells, about 15 to 30 μm in diameter, containing many eosinophilic round granules in the cytoplasm (Fig. 3). We tentatively designated these large cells large granular cells. The granules were also intensively stained with PAS. These granular cells were frequently found around capillaries and near necrotic foci of the tumor. Such large granular cell infiltration was also observed, though to a lesser extent, in the tumors in the mice given rIL-2 or rIFN-α A/D alone on day 17 and day 21 (Table II). These large granular cells were rarely observed in the tumors in the vehicle group. The incidence of large granular cells in the tumors in the mice killed on day 13 is shown in Table III.

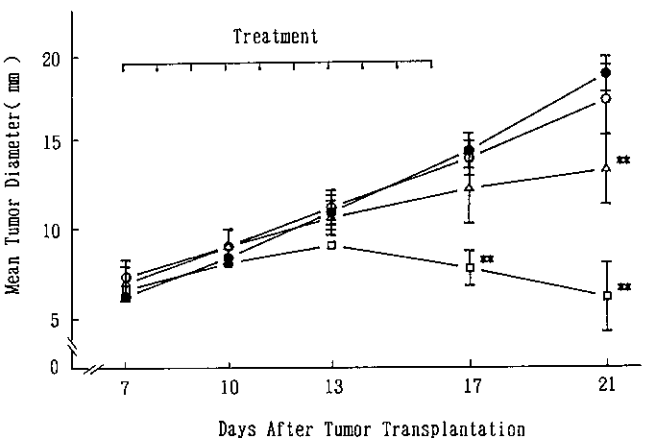


Fig. 1. Changes in mean tumor diameter during treatment. Mice received daily intramuscular injection of vehicle (5% normal mouse serum, ●), rIL-2 (10 μg/mouse, ○), rIFN-α A/D (2×10^5 IU/mouse, △) or rIL-2 plus rIFN-α A/D (□) from day 7 to day 16 after colon 26 tumor transplantation. Means±SD (n=3). **: Significantly different from vehicle group ($P<0.01$), Dunnett's test.

Table II. Histological Changes in Tumors in Mice Treated with rIL-2 and/or rIFN-α A/D

Group	Days after tumor transplantation			
	9	13	17	21
Vehicle				
Tumor necrosis	+	+	+	2+
Infiltration of LGC[a)]	±	±	+	+
rIL-2				
Tumor necrosis	+	+	2+	2+
Infiltration of LGC	±	+	2+	2+
rIFN-α A/D				
Tumor necrosis	+	+	3+	3+
Infiltration of LGC	±	2+	3+	3+
rIL-2+rIFN-α A/D				
Tumor necrosis	+	2+	4+	4+
Infiltration of LGC	±	3+	4+	4+

a) LGC: Large granular cells.
Mice received rIL-2 (10 μg) and/or rIFN-α A/D (2×10^5 IU) from day 7 to day 16. The scores are the mean values of 3 mice in each group. ±, very slight; +, slight; 2+, moderate; 3+, severe; 4+, very severe.

Table I. Tumor Weights in Mice Given Daily Intramuscular Injection of rIL-2 and/or rIFN-α A/D

Group	Tumor weight (mg, mean±SD)			
	Days after transplantation			
	9	13	17	21
Vehicle	184±16	623±42	1455±93	2851±426
rIL-2	195±66	457±110	975±219	1891±306*
rIFN-α A/D	198±74	392±46*	530±72**	856±263**
rIL-2+rIFN-α A/D	147±6	267±22**	227±107**	151±142**

Mice received rIL-2 (10 μg) and/or rIFN-α A/D (2×10^5 IU) from day 7 to day 16 and were killed on days 9, 13, 17 and 21.
*, **: Significantly different from vehicle group (* $P<0.05$, ** $P<0.01$), Dunnett's test.

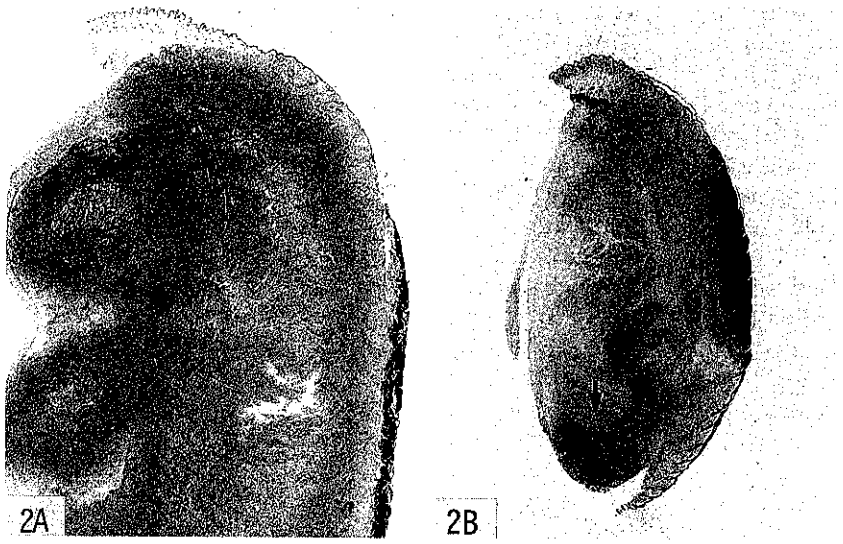


Fig. 2. Colon 26 tumor in mice given vehicle (A) or the combination treatment (B). Tumors were examined on day 17 after transplantation. (A) Tumor tissue is well grown (the score of necrosis: +). (B) Growth of the tumor tissue is very weak. Most of the tumor tissue exhibits necrosis (the score of necrosis: 4+). A small number of tumor cells remains in the periphery of tumor tissue (arrow). Hematoxylin and eosin, ×8.

Table III. Incidence of Large Granular Cells in Tumor

Group	Number of large granular cells/mm² (mean±SD)
Vehicle	3.1±3.1
rIL-2	11.7±6.4
rIFN-α A/D	12.7±0.5
rIL-2+ rIFN-α A/D	47.9±12.1**

**: Significantly different from vehicle group ($P<0.01$), Dunnett's test. Mice received rIL-2 (10 μg/mouse) and/or rIFN-α A/D (2×10^5 IU/mouse) from day 7 to day 12 and were killed on day 13.

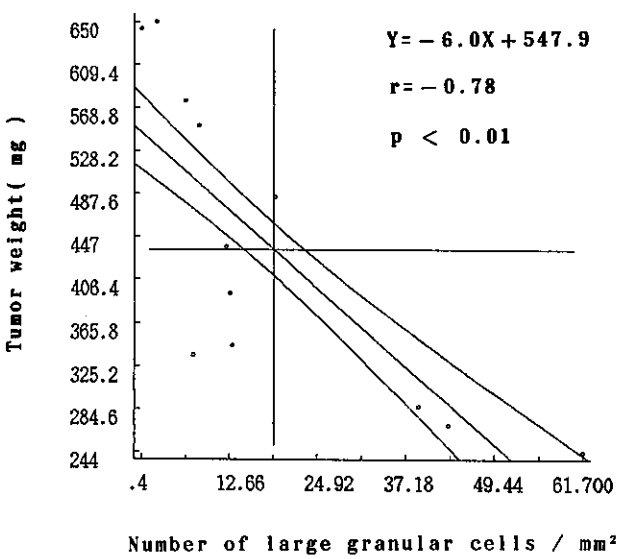


Fig. 4. Correlation between tumor weight and incidence of large granular cells in tumor tissue of mice treated with rIL-2 and/or rIFN-α A/D. Tumors were examined on day 13 after transplantation.

The number of large granular cells was moderately increased (about 4 times) in the mice given rIL-2 or rIFN-α A/D alone and markedly increased (about 16 times) in the mice given the combination treatment. A highly significant correlation was noted between tumor weight and number of the large granular cells on day 13 ($r=-0.78$, $P<0.01$, Fig. 4).

Infiltration of lymphocytes, macrophages and neutrophils was observed to some extent in and around tumors in all groups. However, there were no obvious changes in these cells which seemed to be related to the treatments.

Immunohistochemical findings in the tumor There were many Thy-1-positive large cells in the tumors in the mice given the combination treatment (Fig. 5). These cells were negative for surface IgG. Examination of the adjacent sections revealed that these Thy-1-positive large cells correspond to large granular PAS-positive cells. Lyt-1 or Lyt-2 positive cells were smaller both in number and in size than the Thy-1-positive large cells.

Ultrastructure of large granular cells in the tumor The large granular cells contained abundant round granules about 1 to 2 μm in diameter, well-developed Golgi complexes and clusters of glycogen granules in the cytoplasm (Fig. 6). The cytoplasmic granules were electron-dense

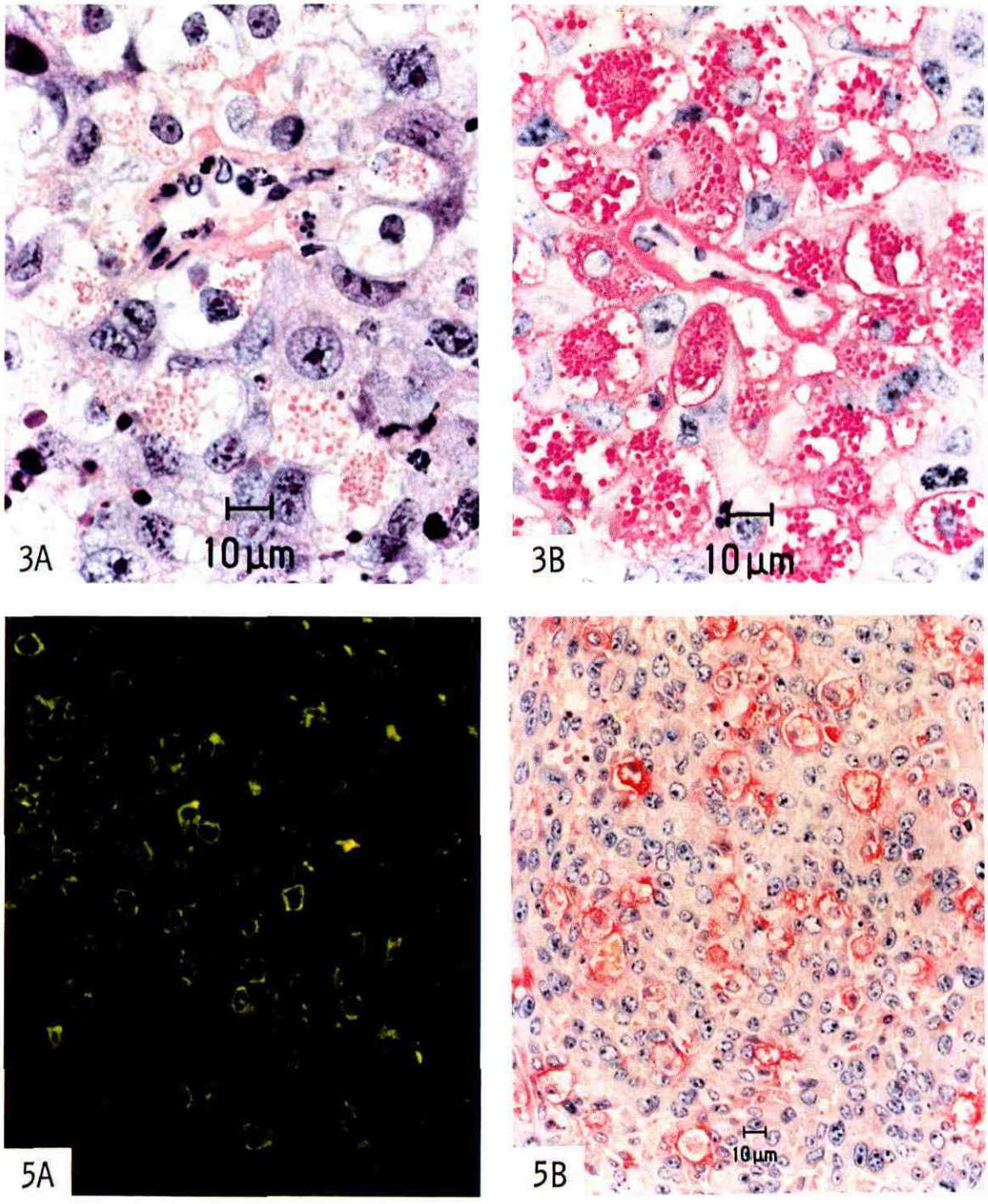
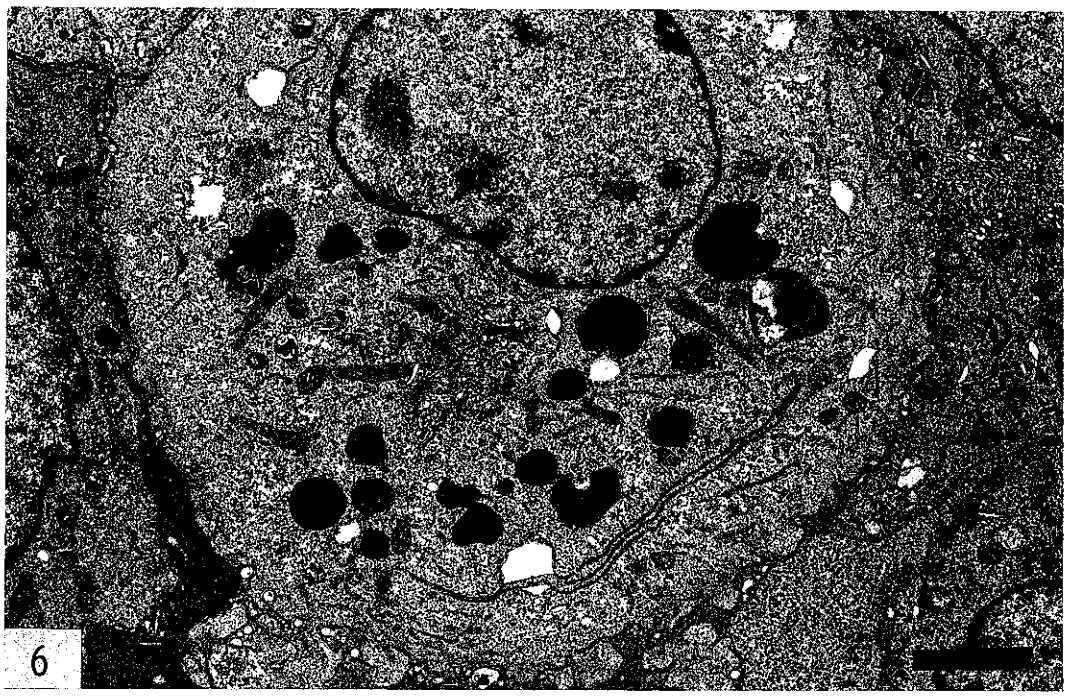


Fig. 3. Colon 26 tumor in mice given the combination treatment. The tumor was examined on day 17. (A) Many large cells containing weakly esosinophilic granules are seen around a blood vessel (the score of infiltration of large granular cells: 4+). Hematoxylin and eosin, ×630. (B) The granules of the large cells are also PAS-positive. PAS, ×630.

Fig. 5. Colon 26 tumor in mice given the combination treatment. The tumor was examined on day 13. The large granular cells are positive for FITC-conjugated anti Thy-1.2 antibody (A) and anti-Thy-1 antibody (B). (A) Direct immunofluorescence method, ×300. (B) ABC-AP method, ×415.



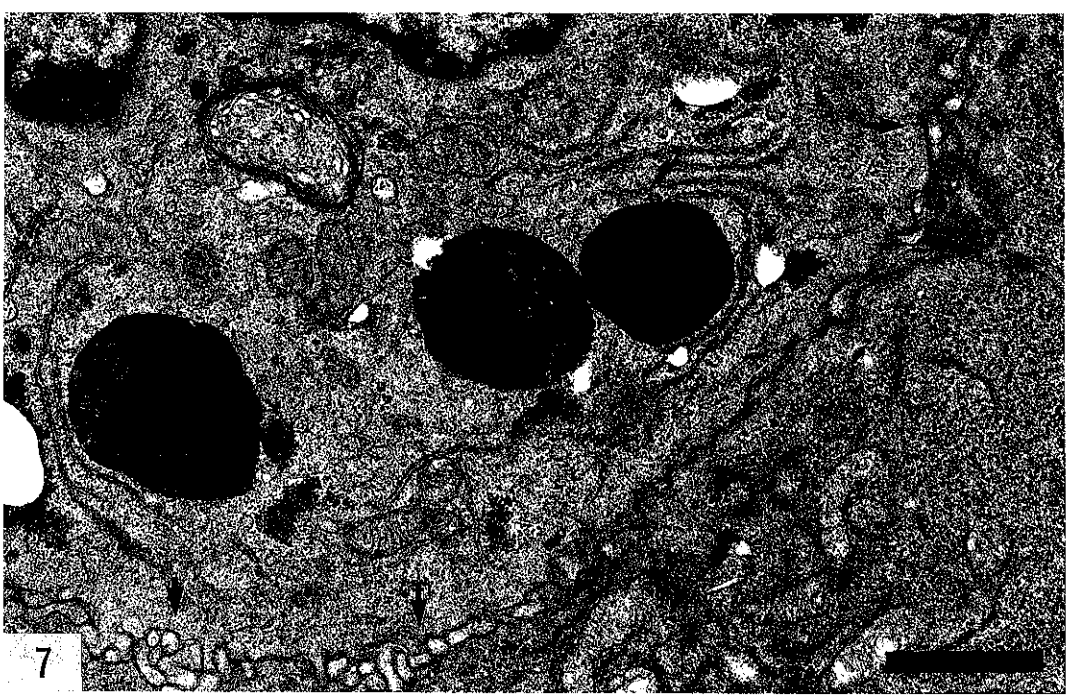


Fig. 6. Electron micrograph of a large granular cell in a tumor from a mouse given the combination treatment. The tumor was examined on day 13. The large cell (about 15 μm in diameter) contains many electron-dense granules and well-developed Golgi complexes in the cytoplasm. The bar represents 2 μm. $\times$7500.

Fig. 7. Electron micrograph of a large granular cell in a tumor from a mouse given the combination treatment. The tumor was examined on day 13. The cytoplasmic granules are surrounded by fine, electron-dense membranous vesicles at the periphery. Clusters of glycogen granules appear frequently. The cytoplasmic membrane of the large cell is interdigitated with the cell membrane of adjacent colon 26 tumor cells (arrows). The bar represents 2 μm. $\times$10500.

and contained fine membranous vesicles on the periphery. Some granules had a partially moth-eaten-like hollow in the matrix. Occasionally, interdigitation of cell membranes of the large granular cells and the adjacent colon 26 tumor cells was observed (Fig. 7).

DISCUSSION

rIL-2[20–29] and rIFN-α A/D[8–10, 30–34] have antitumor and antimetastatic activities *in vivo* in mice. It is hence expected that treatment with the combination of rIL-2 and rIFN-α A/D may have more potent antitumor effects. Brunda *et al.*[2] reported that the combination treatment prominently inhibited tumor growth and spontaneous metastases in murine tumor models. They observed the induction of an NK-like cell population in the liver. Such a cell population has been shown to enhance cytotoxic activity and correlate well with anti-tumor activity *in vivo*. Iigo *et al.*[3] also observed evident antitumor effects of the combination of rIL-2 and either rIFN-α A/D or murine rIFN-β. These combination treatments significantly suppressed the growth of tumors such as Adenocarcinoma 755, B16-F10 Melanoma, colon 38 and colon 26. They suggested that T cell immunity may be important in the regression of the tumors, because of the lesser potentiation of antitumor activity in athymic mice. However, there was no histological evidence regarding the tumor histology in these reports. Therefore, from these reports it is not certain how T-cell immunity or NK-like cells attack the tumor cells.

The present study confirmed the prominent synergistic antitumor effect of rIL-2 in combination with rIFN-α A/D on the growth of subcutaneously established colon 26 in mice. Histologically, tumor necrosis and infiltration of large granular cells were most evident in the regressing tumors in the combination group. On day 13, there was a significant correlation between the number of infiltrating large granular cells and the tumor weight in mice treated with rIL-2, rIFN-α A/D and rIL-2 in combination with rIFN-α A/D. It is very interesting that reduction in tumor weight developed after day 13 in mice that received the combination treatment. Since there were no other pathologic changes in the tumor which suggest a cause of the tumor necrosis, it is likely that the infiltration of large granular cells contributed to the tumor necrosis and reduction in tumor mass. These large granular cells in the tumors appear to be the first morphological evidence of the cytological entity of the antitumor effect of the combination treatment *in vivo*. In addition, it is also important to note that the number of cells which infiltrated in the tumors in either the rIL-2 or rIFN-α A/D group had a tendency to increase.

Immunocytochemically, the granular cells possessed Thy-1 surface antigen but not Lyt-1, Lyt-2 or IgG surface antigens. The morphologic features of the large granular cells were very distinct: large cell size (15–30 μm in diameter) and abundant cytoplasmic granules intensely stained with PAS. The size of the granular cells was noticeably larger than that of resting large granular lymphocyte (LGL) and T cells, and these cells appeared to be different from the lymphoid cells which proliferated in tissuses of normal mice given rIL-2 intraperitoneally.[12] It is well known that LGL-like murine cell lines which are maintained *in vitro* in a medium containing IL-2 develop marked natural killer activity. These LGL-like cells possess Thy-1-positive surface antigens[35–39] and PAS-positive cytoplasmic granules.[35]

Ultrastructurally, LGL-like cells contain well-developed Golgi complex, a considerable quantity of glycogen granules and electron-dense granules enveloped in fine vesicular bodies in the cytoplasm.[35–37] Murine LAK cells were also characterized as having Thy-1 surface antigen and cytoplasmic granules.[40] The present large granular cells in the colon 26 tumor were very similar to those cell lines with NK activity and LAK cells *in vitro* with respect to Thy-1 surface antigen, the morphological features of the cytoplasmic granules and cell size. Recently, Hook *et al.*[41] reported a morphological study with LAK cells conjugated with human tumor cells *in vitro*. They found that the LAK-tumor cell conjugates formed very tight plasma membrane bonds with numerous interdigitations of the cells. We also found similar interdigitation of large granular cells and colon 26 tumor cells in the tumors in the mice given the combination treatment. Accordingly, it is probable that the present large granular cells in the colon 26 tumors are a type of activated or highly differentiated lymphoid cells. The small amount of infiltration of the large granular cells in the vehicle group suggests that infiltration of these granular cells is one of the normal host reactions in mice bearing the tumor, and it is likely that the combination treatment may augment this host reaction.

The origin of the large granular cells is still unknown. The fact that many granular cells exist near the capillaries suggests the possibility that these cells are transported by the blood stream directly. However, it is conceivable that precursor cells delivered via the blood may differentiate into granular cells *in situ*.

In summary, the combination treatment with rIL-2 and rIFN-α A/D produces extensive tumor necrosis accompanied with marked infiltration of large granular cells. Although we could not obtain direct functional evidence for the antitumor action of the large granular cells, they are considered to play important roles in the tumor regression by strengthening host immunity in response to the combination treatment. Therefore, the combination treatment with rIL-2 and rIFN-α may become an efficacious regimen for the clinical treatment of cancer.

ACKNOWLEDGMENTS

We are greatly indebted to Dr. Akira Imada of the Biology Research Laboratories for his encouragement and continued interest in this work. We greatly appreciate the skillful technical assistance of Mr. Kazunori Gotoh and Mrs. Emiko Oda.

(Received April 27, 1989/Accepted July 8, 1989)

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