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Enhancement of LAK-like activity and cytokine induction in regional lymph nodes and spleen cells of mice after intralymphnodal injection of OK-432, a killed streptococcal preparation

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A single dose of inactivated streptococci (OK-432) was injected into the popliteal lymph nodes of male CDF$_1$ mice and its effects on popliteal, inguinal, and para-aortic lymph node cells and spleen cells were investigated and compared with the effects of subcutaneous injections of the same dosage of OK-432. Regional lymph node cells and spleen cells obtained from intralymphnodally injected mice lysed not only natural killer (NK)-sensitive YAC-1 cells, but also NK-resistant P-815 and meth-A cells. Lysis of target cells was inhibited when effector cells were treated with anti-Thy-1.2 or anti-Lyt-2.2 monoclonal antibody and complement, but no inhibition was apparent after treatment with anti-asialo-GM1 or anti-Lyt-1.2 antibody and complement. These results suggest that the effector cells are lymphocyte-activated killer (LAK) cells. An enhanced capacity of lymph node cells to produce cytokines, tumor necrosis factor and interleukin 1 upon restimulation with lipopolysaccharide was found only in intralymphnodally injected mice. Thus, the induction of LAK-like cells and cytokine production in regional lymph nodes and spleen cells by the intralymphnodal administration of OK-432 should be effective for the inhibition or treatment of lymph node metastases.

Key words: Biological response modifiers, cytokine induction, interleukin 1, intralymphnodal injection, LAK-like cells, OK-432, tumor necrosis factor.

Introduction

A bacterial immunopotentiator OK-432, a killed streptococcal preparation, has been used extensively as a therapeutic agent for malignant disease in humans and experimental animals, and a number of successful results have been reported.

The immunomodulatory effects of OK-432 have been investigated widely, including its ability to: augment natural killer cells (NK), lymphokine-activated killer cells (LAK) and macrophages; act as an adjuvant for the induction of cytotoxic T-lymphocytes (CTL) with tumor vaccines; and induce various cytokines.[1–5] In clinical studies and animal experiments, OK-432 has been found to augment these activities in peripheral blood lymphocytes (PBL), pleural effusion cells, spleen cells and regional lymph node cells.[1–5]

Although the administration regimen for OK-432 has been studied extensively, the most desirable administration schedule and site are still unknown. Intraperitoneal and subcutaneous injections of OK-432 are performed commonly, but the therapeutic effect of a single intraperitoneal or subcutaneous injection is poor, resulting in the need for frequent dosing for long periods of time.

Advanced gastrointestinal tract cancers tend to recur locally or systemically with distant metastases and peritoneal dissemination occurring even if a curative macroscopic operation was performed. The recurrence rate is often related to the presence or absence of lymph node metastases.[6,7] Several studies have suggested an impairment of cell-mediated immunity, especially of T lymphocyte function, in peripheral blood and regional lymph nodes of cancer patients.[8,9] These immune dysfunctions were shown to be closely correlated with tumor burden, clinical stage and response to therapy.

It is commonly considered that the NK and LAK activities of regional lymph node cells are very low,[8,9] and that enhancing the immunological response in the regional lymph nodes by local immunotherapy will destroy circulating tumor emboli and prevent metastatic spread in the lymph nodes.

We have shown previously in animal experiments that intralymphnodal administration of OK-432 is effective against lymph node metastases,[10] but little

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is known about the immunomodulatory effect of OK-432 administered directly into the lymph nodes on the immunopotential of regional lymph node cells. In the present study we investigated the effect of intralymphnodal administration of OK-432 on cytotoxic activity and cytokine induction in the regional lymph nodes and spleen.

Materials and methods

Mice and tumor cell lines

Male, pathogen-free, CDF_1 mice, 6–8 weeks old, were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The target cells with different NK-sensitivities used for the *in vitro* cytotoxicity test were mouse sarcoma meth-A, mouse mastocytoma P-815 and mouse lymphoma YAC-1 cells. The target cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) medium supplemented with 10% heat-inactivated fetal bovine serum and 60 μg/ml kanamycin (complete medium) in humidified air with 5% CO_2. All experiments were performed under these conditions.

Intralymphnodal injection of OK-432 and preparation of effector cells

OK-432, an antitumor agent also known as Picibanil, was kindly donated by the Chugai Pharmaceutical Co. (Tokyo, Japan). OK-432 was suspended in saline solution at a concentration of 2 mg/ml, and 0.025 ml intralymphnodal and subcutaneous injections were administered on day 0. Popliteal lymph nodes, which are about 2.5 cm away from the foot-pads of mice, are very small and difficult to locate. To make them easier to find, methylene blue solution was injected subcutaneously into the foot-pad of the left hind paw. Popliteal lymph nodes were stained within a few minutes after this subcutaneous injection. Intralymphnodal injections were then made under general pentobarbital anesthesia (OK-432 I.L. group). Subcutaneous injections of OK-432 and saline into the foot-pad of control mice followed the same schedule (OK-432 S.C. group and saline group).

On days 0, 1, 4, 7 and 10, after administration of OK-432 or saline, 10 mice at each time point were killed and popliteal lymph nodes, inguinal lymph nodes, paraaortic lymph nodes and spleens were excised, homogenized and single cell suspensions were prepared.

The spleen cells were treated with ammonium chloride–Tris buffer solution for 1 min at room temperature to lyse red blood cells, then adherent cells in the whole lymph node cells and spleen cells were removed by incubation in medium in a tissue culture flask (Costar) at 37°C for 1 h. These non-adherent cells served as effector cells. The effector cells were counted and prepared in complete medium to a concentration of 10^7/ml.

Chronological changes of cytotoxicity in regional lymph node cells and spleen cells after administration of OK-432

Cell-mediated cytotoxicity was assayed by the 4 h chromium release method,[11] using YAC-1, P815 and meth-A as target cells. Target cells (1×10^6 cells) were incubated with 100 μCi $NaCrO_4$ for 1 h at 37°C, washed three times with minimal essential medium (MEM) and resuspended in complete medium at a concentration of 1×10^5 cells/ml. Target cell suspensions (100 μl) were incubated with equal volumes of 1×10^7 effector cells/ml of effector cell suspensions for 4 h at 37°C in a round-bottomed 96-well microtiterplate. Samples of supernatant (100 μl) were then harvested and counted in a gamma counter (Pakkard-Auto Gamma). Spontaneous release (background), computed from wells containing target cells and complete medium only, was consistently below 5% of the total counts obtained from wells containing target cells lysed with 2% sodium dodecyl sulfate. Three wells were taken for each data point. The percentage of specific cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = \frac{(\text{test c.p.m.} - \text{spontaneous c.p.m.}) \times 100}{\text{maximum c.p.m.} - \text{spontaneous c.p.m.}}$$

Cell depletion with antibody and complement

The lymphocytes and splenocytes obtained from OK-432 intralymphnodally injected mice were collected in cold MEM, washed once with MEM and suspended in serum-free RPMI 1640 medium at a concentration of 2×10^7 cells/ml. Then, 1 ml of cell suspension was incubated for 30 min at 37°C with either monoclonal anti-Thy-1.2

(Olac, clone F7D5, Bicester, England), anti-Lyt-2.2 or anti-Lyt-1.2 (Becton-Dickinson Immunochemistry Systems, Oxnard, CA, USA) at a 1/500 dilution or anti-asialo-GM1 (Wako Pure Chemical, Osaka, Japan) at a 1/50 dilution. Incubation was continued for another 45 min at 37°C with a low-toxicity rabbit complement (Cederlane Laboratories Ltd., Ontario, Canada) at a 1/10 dilution. Treated cells were washed twice with MEM and dead cells were counted by Trypan blue dye exclusion. Reconstitution for the cytotoxicity assay, however, was made with the original volume of medium.

Cytokine assays

Two different cytokines, interleukin (IL)-1 and tumor necrosis factor (TNF), were assayed. On day 1 after intralymphnodal or subcutaneous injection of either OK-432 or saline, the popliteal, inguinal and para-aortic lymph node cells were prepared. These cells were cultured in medium containing 10% fetal calf serum and 5 μg/ml lipopolysaccharide, and the resulting culture supernatants were assayed for cytokines. IL-1 alone has been shown to be highly mitogenic for peanut agglutinin-negative thymocyte populations. Therefore, IL-1 activity was assayed by measuring the mitogenic activity of the samples on a murine thymocyte population.[12–14] Briefly, peanut agglutinin-negative thymocytes from young C3H/HeJ mice were suspended in 0.1 ml of medium (5×10^5 cells/well) and cultured with 0.1 ml of diluted samples in a 96-well microplate at 37°C in a CO_2 incubator. After 48 h, 0.5 μCi [^{3}H]thymidine was added and the incubation continued for 16 h before determining c.p.m. by the method of Mizel *et al.*[12]

TNF activity in the test samples was determined in a cytotoxic assay using TNF-sensitive mouse fibroblast L929 cells in the presence of 1 μg/ml actinomycin D.[15,16] Briefly, aliquots of culture supernatant were added to a L929 cell monolayer cultured in plastic flat-bottomed 96-well microplates. The cell mixtures were incubated for 20 h in the presence of 1 μg/ml actinomycin D. After incubation, the cultured L929 cells were stained with crystal violet and the absorbance determined with a micro-ELISA autoreader. One unit of TNF activity was defined as the reciprocal of the dilution necessary for lysis of 50% of the L929 cells. Human recombinant TNF-α (Dainippon Seiyaku Inc., Japan) was used as an internal standard in each assay. Murine anti-TNF-α monoclonal antibody (IgG1) (Midori Juji Inc., Osaka, Japan) was used to confirm that the cytotoxic activity against L929 cells was due to TNF. Polyclonal rabbit anti-mouse IL-1-α (Genzyme, Boston, MA) was also used to confirm the presence of true IL-1.

Statistical analysis

Differences in cytotoxicity and cytokine induction were evaluated by Student's *t*-test.

Results

Chronological changes of cytotoxicity in the regional lymph node and spleen cells after intralymphnodal administration of OK-432

Cytotoxicity was measured on days 0, 1, 4, 7 and 10 following the intralymphnodal or subcutaneous injection of ether OK-432 or saline. After intralymphnodal injection, the cytotoxicity of the cells in both regional lymph nodes and spleen increased, as shown in Figure 1(a–d) for YAC-1 cells. The highest level of cytotoxicity for popliteal lymph node, inguinal lymph node and spleen cells was observed on day 1. Para-aortic lymph node cells exhibited the highest toxicity on days 1 and 7. The cytotoxicity of popliteal lymph node, para-aortic lymph node and spleen cells on days 1, 4, 7 and 10 was statistically higher in the OK-432 I.L. group than in the other groups ($p < 0.05$). As for the inguinal lymph node cells, their cytotoxicity was significantly greater than in the other groups only on day 1 in the OK-432 I.L. group ($p < 0.05$).

Target cell specificity of effector cells

To determine the target cell specificity of killer cells, NK-sensitive and NK-resistant cell lines were used as target cells. As shown in Figure 2, regional lymph node cells and spleen cells from mice injected intralymphnodally with OK-432 showed cytotoxic activity not only against YAC-1, but also against P815 and meth-A.

Cell depletion with antibody and complement

Since lymph node cells and spleen cells from OK-432 intralymphnodally injected mice showed

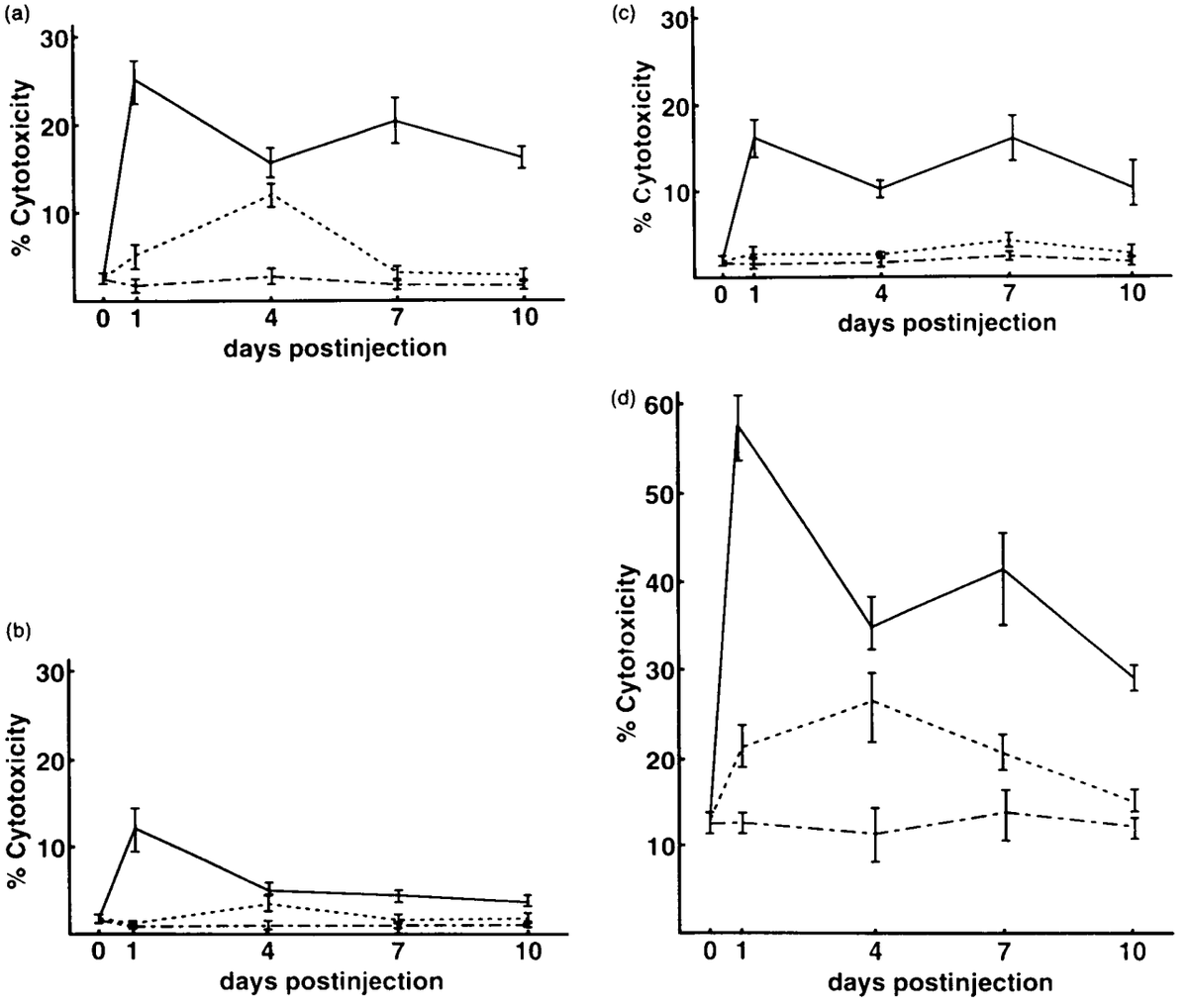

Figure 1. Chronological changes in the cytotoxicity of the cells in (a) popliteal lymph nodes, (b) inguinal lymph nodes, (c) para-aortic lymph nodes and (d) spleen on days 0, 1, 4, 7 and 10 following the intralymphnodal or subcutaneous injection of either OK-432 or saline. Assays were performed in triplicate at non-adherent lymph node cells or non-adherent spleen cells to target cell ratio of 100:1. Each point and vertical bar shows the mean ±SD of triplicate determinations from three experiments. (—) OK-432 I.L., (- - -) OK-432 S.C.; (– – –) saline.

definite cytotoxicity against YAC-1, P815 and meth A cells, further characterization of these killer cells was made. Regional lymph node and spleen cells were obtained on day 1 from five or six mice in each treated group, and then incubated with either anti-Thy-1.2, anti-Lyt-2.2, anti-Lyt-1.2 or anti-asialo-GM1 and complement in order to determine the phenotype of the killer cells. Lysis of NK-sensitive YAC-1 cells was inhibited when effector cells were treated with an anti-Thy-1.2 or anti-Lyt-2.2 monoclonal antibody and complement, whereas anti-asialo-GM1 or anti-Lyt-1.2 antibody and complement produced little inhibition. Cytotoxic activities directed against NK-resistant P815 cells followed almost the same pattern. The target specificity of these killer cells is therefore Thy-1.2$^+$, Lyt-2.2$^+$, Lyt-1.2$^-$ and asialo-GM1$^-$; this coincides with the cytotoxicity profile of LAK cells (Figure 3).

Cytokine assays

IL-1 production by OK-432 I.L. popliteal lymph node cells was significantly higher than that of any other group of cells ($p < 0.05$), although cells from

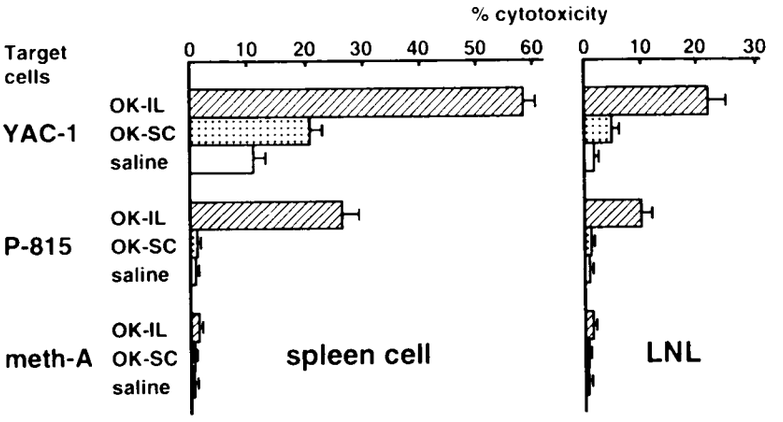

Figure 2. Target cell specificity of effector cells on day 1 after intralymphnodal or subcutaneous injection of OK-432. Effector cells were non-adherent spleen cells or non-adherent popliteal lymph node cells. Cytotoxicity against various target cells was measured at an effector to target cell ratio of 100:1. Each point and vertical bar shows the mean ±SD of triplicate determinations from three separate experiments. LNL, lymph node lymphocyte.

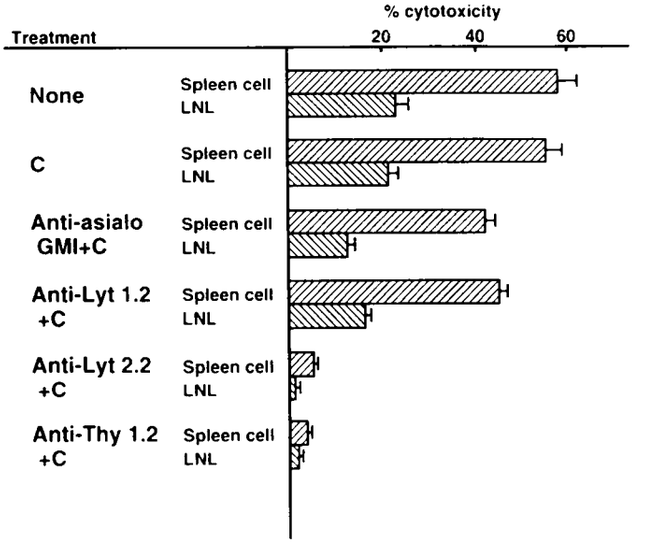

Figure 3. Phenotypic expression of effector cells in popliteal lymph nodes and spleen induced by intra lymphnodal injection of OK-432. Effector cells in the popliteal lymph nodes and spleen were sensitive to treatment with anti-Thy-1.2 or anti-Lyt-2.2 monoclonal antibody and complement, but only slightly sensitive to anti-Lyt-1.2 antibody or anti-asialo-GM1 antibody and complement. Each point and horizontal bar shows the mean ±SD of triplicate determinations from three separate experiments. LNL, lymph node lymphocyte.

both OK-432 I.L. and OK-432 S.C. groups produced more cytokines than the untreated group of cells (Figure 4a).

TNF activity was detected in the OK-432 I.L. popliteal and para-aortic lymph node cells and in the popliteal lymph node cells of the OK-432 S.C. group. TNF activity was detected in OK-432 I.L. popliteal and para-aortic lymph node cells was significantly higher than that of any other group of cells ($p < 0.05$). No activity was detected

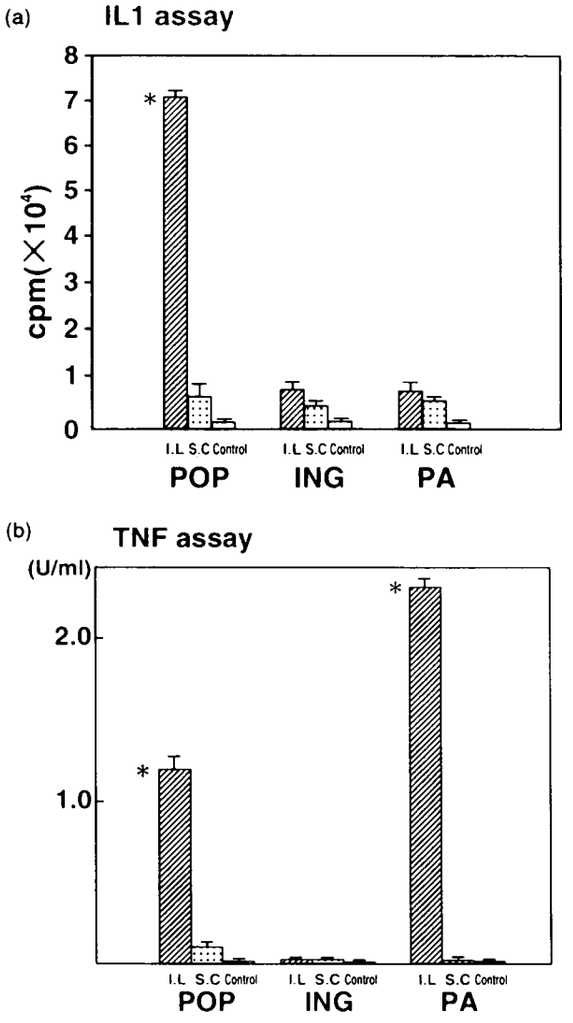

Figure 4. Cytokine production (a, IL-1; b, TNF) in culture supernatants was analyzed on day 1 following the intralymphnodal or subcutaneous injection of either OK-432 or saline. All incubations were performed in triplicate and cytokine activity was analyzed for each supernatant. Representative results from three separate experiments are depicted. Data are represented as means ±SD. $^*p < 0.05$ versus untreated controls. POP, popliteal; ING, inguinal; PA, para-aortic.

from any other sample (Figure 4b). Untreated control and saline group showed almost the same results. TNF activity was completely blocked by the antibody to TNF-α (>90%). IL-1 activity was also blocked by the antibody to IL 1-α (>80%).

Discussion

The identification of biological response modifiers (BRM) with therapeutic activity against established metastatic disease, optimization of the therapeutic protocol and elucidation of the mechanism of therapeutic activity are the major priorities for preclinical and clinical studies for these agents. Earlier studies revealed the importance of dosage, route, duration, and schedule of administration in optimizing the therapeutic activity of BRM in the treatment of neoplastic disease.[17–19]

Lymph nodes normally lack spontaneous NK activity,[9] although the subcutaneous injection of a BRM such as *Corynebacterium parvum* into the area around the regional lymph nodes results in strong activation of their cytotoxicity activity as shown by the lysis of YAC-1 and relatively NK-resistant tumor cells.[20,21] However, the effects of the intralymphnodal injection of OK-432 have not been reported. In this study we have succeeded in enhancing the cytotoxicity in the regional lymph nodes and spleen by intralymphnodal injection of OK-432.

We have used the monoclonal antibody and complement depletion technique to characterize the cell surface phenotype of cells activated by OK-432. It is commonly considered that LAK effector cells are sensitive to treatment with anti-Thy-1.2 or anti-Lyt-2.2 monoclonal antibody and complement, but only slightly sensitive to anti-Lyt-1.2 antibody and complement.[23–25] NK cells are thought to express Lyt-1 and low levels of Thy-1.[24,25] Another important difference between LAK cells and NK cells is the ability of LAK cells to kill a variety of fresh tumor target cells that are resistant to NK cell lysis. Effector cells in the regional lymph nodes and spleen were sensitive to treatment with anti-Thy-1.2 or anti-Lyt-2.2 monoclonal antibody and complement, but only slightly sensitive to anti-Lyt-1.2 antibody or anti-asialo-GM1 antibody and complement, suggesting the majority of the cytotoxicity cells are not NK cells, but LAK cells.

An interesting characteristic of LAK cells is that when they are propagated *in vitro* in the presence of IL-2, they exhibit strong cytotoxic activities towards a variety of fresh or cultured syngeneic, allogeneic and xenogeneic tumor cells, but not towards spleen lymphocytes. LAK cells have been used for the treatment of malignant disease in humans and experimental animals, and some successful results have been reported.[23,26] It is commonly considered that culturing in the presence of IL-2 is necessary to produce LAK cells. However, our results indicated that only a single intralymphnodal administration of OK-432 can induce LAK-like cells in the regional lymph nodes as well as in the spleen.

OK-432 is considered to be a multi-cytokine inducer.[2] IL-1 can enhance T cell and B cell functions, including cytotoxic T lymphocyte activity,[11,13,14] whereas TNF has a direct antitumor activity.[5,15,16] In this study, an enhanced capacity of lymph node cells to produce cytokines, TNF and IL-1, upon restimulation with lipopolysaccharide was found in the OK-432 I.L. group. This treatment should be effective against lymph node metastases.

The studies reported herein revealed that the intralymphnodal injection of OK-432 is the optimal administration route and should be an important component of an effective OK-432 regimen for the inhibition or treatment of lymph node metastases. These findings suggest that the intralymphnodal administration of OK-432 can augment the local immune response in the regional lymph nodes and also enhance the host's systemic resistance. On the basis of its potent cytotoxicity and cytokine induction ability, we believe that the superior therapeutic effect of intralymphnodal injection of OK-432 against lymph node metastases is related to its immunostimulatory effect in the lymph nodes. However, the actual mechanism of OK-432-induced cytotoxic action is still not clear. To date we have only examined non-specific effector functions and experiments are now under way to further clarify the mechanism of effector cell induction.

Clinically, intralymphnodal inection of OK-432 should have superior therapeutic effects against lymph node metastases and should augment the immune response in the regional lymph nodes. These properties will be valuable in the establishment of new immunotherapeutic protocols against lymph node metastases.

References

1. Hojo H, Hashimoto Y. Cytotoxic cells induced in tumor-bearing rats by a streptococcus preparation (OK-432). *Gann* 1981; **72**: 692–9.
2. Ichimura O, Suzuki S, Saito M, *et al.* Augmentation of interleukin 1 and interleukin 2 production by OK-432. *Int J Immunopharmacol* 1985; **7**: 263–71.
3. Ishii Y, Yamaoka H, To K, *et al.* Inhibition of tumor growth *in vivo* by macrophages from rats treated with streptococcal preparation, OK-432. *Gann* 1976; **67**: 115–22.
4. Vinzenz K, Matejka M, Watzek G, *et al.* Modulation of NK activity in regional lymph nodes by preoperative immunotherapy with OK-432 in patients with cancer of the oral cavity. *Cancer Detect Prev Suppl* 1987; **1**: 463–75.
5. Nara K, Odagiri H, Fujii M, *et al.* Increased production of tumor necrosis factor and prostaglandin E_2 by monocytes in cancer patients and its unique modulation by their plasma. *Cancer Immunol Immunother* 1987; **25**: 126–33.
6. Inokuchi K, Hattori T. Postoperative adjuvant chemotherapy for gastric carcinoma—analysis of data on 1805 patients followed for 5 years. *Cancer* 1984; **53**: 2393–403.
7. Black MM, Oper SR, Speer FD. Microscopic structure of gastric carcinomas and their regional lymph nodes in relation to survival. *Surg Gynec Obstet* 1954; **98**: 725–35.
8. Vose BM, Moore M. Suppressor cell activity of lymphocytes infiltrating human lung and breast tumors. *Int J Cancer* 1979; **24**: 579–90.
9. Vose BM, Vanky F, Arvog S. Natural cytotoxicity in man: activity of lymph node and tumor infiltrating lymphocytes. *Eur J Immunol* 1977; **7**: 753–64.
10. Okano S, Sawai K, Fujioka T, *et al.* Intralymphnodal injection of OK-432 for treatment of lymph node metastases. *Jpn J Cancer Chemother* 1992; **19**: 1598–600 (in Japanese).
11. Herberman RB, Nunn ME, Holden HT, *et al.* Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. Characterization of effector cells. *Int J Cancer* 1975; **16**: 230–6.
12. Mizel SB. Interleukin 1 and T cell activation. *Immunol Rev* 1982; **63**: 1887–921.
13. Pools S, Graines Das RE. The international standards for interleukin-1 alpha and interleukin-1 beta. Evaluation in an international collaborative study. *J Immunol Methods* 1991; **142**: 1–13.
14. Hogquist KA, Unanue ER, Chaplin DD. Release of IL-1 from mononuclear phagocytes. *J Immunol* 1991; **147**: 2181–6.
15. Branch DR, Shah A, Guilbert LT. A specific and reliable bioassay for the detection of femtomolar levels human and murine tumor necrosis factors. *J Immunol Methods* 1991; **143**: 251–61.
16. Baarsch MJ, Wannemuehler MJ, Molitor TW, *et al.* Detection of tumor necrosis factor alpha from porcine alveolar macrophage using an L929 fibroblast bioassay. *J Immunol Methods* 1991; **140**: 15–22.
17. Talmadge JE, Fidler IJ, Oldham RH. *Screening for the biological response modifiers: methods and rationale.* Boston: Martius Nijhoff 1985: 1–193.
18. Ono T, Kurata S, Wakabayashi K, *et al.* Inhibition effect of a streptococcal preparation (OK-432) on the nucleic acid synthesis in tumor cells *in vitro*. *Gann* 1973; **64**: 59–71.
19. Oshimi K, Kano S, Takasu F, *et al.* Augmentation of mouse natural killer cell activity by a streptococcal preparation, OK-432. *J Natl Cancer Inst* 1980; **65**: 1267–80.
20. Miyata H, Himeno K, Nomoto K. Mechanisms of the potentiation of specific antitumor immunity by intratumor injection of *Corynebacterium parvum*. *Cancer Res* 1983; **43**: 4670–5.
21. Johnson TR, North RJ. Frequency analysis of augmented CTL production associated with *Corynebacterium parvum*-induced tumor regression. *Immunology* 1987; **60**: 361–6.
22. Kedar E, Ikejiri BL, Gorelik E, *et al.* Natural cell-mediated cytotoxicity *in vivo* and inhibition of tumor growth *in vivo* by murine lymphoid cells cultured with T cell growth factor (TCGF). *Cancer Immunol Immunother* 1982; **132**: 3205–20.
23. Rosenstein M, Yron I, Kaufmann Y, *et al.* Lymphokine-activated killer cells: lysis of fresh syngeneic natural

killer-resistant murine tumor cells by lymphocytes cultured in interleukin 2. *Cancer Res* 1984; **44**: 1946–84.
24. Gimcher L, Shen FU, Cantor H. Identification of a cell-surface antigen expressed on natural killer cells. *J Exp Med* 1977; **145**: 1–9.
25. Kiesling R, Klein E, Wigzel H. 'Natural' killer cells in the mouse. 1. Cytotoxic cells with specificity and distribution according to genotype. *Eur J Immunol* 1975; **5**: 112–7.
26. Rosenberg SA. Adoptive immunotherapy of cancer. *Cancer Treat Rep* 1984; **68**: 233–45.

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