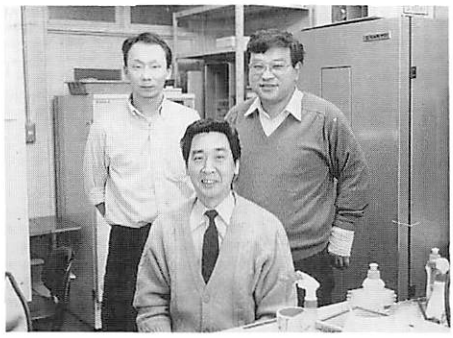
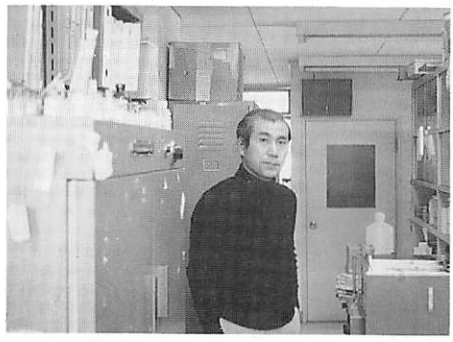
SCIENTIFIC REPORTS

Experimental Therapeutics





DEPARTMENT OF EXPERIMENTAL THERAPEUTICS

GENERAL SUMMARY

There have been some changes in this department. Dr. Saburo Koshimura, the professor of the department, retired in March 1986. Dr. Takuma Sasaki, who was working at the time at the National Cancer Research Institute, was appointed professor of the department in October 1986. Dr. Motohiro Tanaka joined us as a research associate in February 1987.

A wide range of research in the fields of fundamental and applied chemotherapy is done in this department, as is cooperative work with other institutions. Our research projects and present activities are briefly introduced below.

1. *Development of new antitumor substances and biological response modifiers (BRM) from naturally occurring products and organic synthetic compounds, involving screening methods*

Active anticancer substances are being searched for among marine products (scallop, abalone, sea urchin, etc.), mushrooms, microorganisms, metal complexes, and heterocyclic compounds.

2. *Development and clinical application of chemosensitivity tests with use of chick embryo*

Methods that use chick embryos have many advantages for chemosensitivity tests and also in the search for new anticancer drugs. Chick embryos are useful for studies of the growth and metastasis of tumors and the response of tumors to anticancer drugs.

3. *Studies on host defense mechanisms and augmentation of antitumor host factors*

Elucidation of the mechanisms of action and clarification of the structure-activity relationships of antitumor substances and BRM is aimed at. Based on knowledge of mechanisms, development of a strategy for augmentation of antitumor host defenses, elimination of immunosuppressive host factors, etc., can begin.

4. *Studies of cancer treatment and prevention*

Development of the most effective methodology to prevent and to cure cancer within a multidisciplinary framework involves the study of the best combination of surgery, radiotherapy, hyperthermia, and chemotherapy.

These studies were supported in part by Grants-in-Aid (61010096, 62010033, and 62010049) for Cancer Research from the Ministry of Education, Science, and Culture, Japan, the Japanese Foundation for the Multidisciplinary Treatment of Cancer, and the Hokkoku Foundation for Cancer Research.

(1) Development of new anticancer drugs.

T. Sasaki, M. Tanaka, and Y. Endo[1)]

1. Antitumor macromolecular fraction from marine animals.

This study of the stimulation of host defense mechanisms against experimental tumors has for several years been part of a broad program that seeks agents that stimulate the host's immunologic defenses against neoplastic growths. Most antitumor agents of known structure are small to medium-size molecules of low molecular weight. However, some macromolecular substances have attracted interest because of their antitumor activity and their relatively low toxicity.

The macromolecule fractions from the aqueous extracts of 27 marine animals were tested for their antitumor activity against murine transplantable sarcoma 180, solid form, in ICR mice. The macromolecule fractions in the shellfish category, such as *Haliotis discus hannai, H. diversicolor aquatilis, Batillus cornutus, Patinopecten yessoensis, Placopecten magellanicus, Aequipecten irradians, Mercenaria mercenaria, Mya arenaria,* and *Crossostrea virginica,* inhibited the growth of sarcoma 180 with inhibition ratios of 80% or more. Aqueous extracts from *Strongylocentrotus nudus, Halocynthia hilgendorfi f. ritteri, Styela plicata, Ecteinascidia turbinata, Didemnum varians,* and *Megabalanus rosa* also had inhibition ratios of 60% or more. Various doses, schedules, and administration routes were not tested for each fraction, so the fractions may not have been used under optimum conditions in this study. Nevertheless, we concluded that the macromolecule fractions from the extracts of these marine animals had surprisingly strong antitumor effects on sarcoma 180. These results show that marine animals are promising sources for development of new anticancer drugs.

The extracts of *P. yessoensis* and *H. discus hannai* were fractionated by use of Diaflo membranes, DEAE-Sepharose CL6B, DEAE-Sephacel, and Sephadex G-75. The partly purified fraction from *P. yessoensis* (DC-1) consisted of 16.8% carbohydrate, 64.5% protein, 2.7% lipid, and other compounds, and its relative molecular mass was about 90,000 daltons. The fraction from *H. discus hannai* (Fraction 9) was not completely purified, but its major component was glycoprotein with 22% carbohydrate and 44% protein. Its relative molecular mass was estimated to be about 290,000 daltons by gel filtration. Both fractions strongly inhibited the growth of sarcoma 180 in ICR mice. Both fractions were digested by pronase E and also treated with sodium metaperiodate. The antitumor activity of DC-1 disappeared upon treatment with sodium metaperiodate and that of Fraction 9 was also lost by digestion with pronase E. These results showed that the active component of DC-1 was carbohydrate, and that of Fraction 9 was protein. These fractions did not have any antitumor activity in T-cell-deficient nude mice, and they activated the

cytostatic activity of peritoneal and alveolar macrophages *in vivo*. They did not have a direct cytotoxic effect *in vitro*. This suggests that the antitumor activity of these fractions is due to stimulation of host-mediated responses.

2. Antineoplastic compounds from marine animals.

a) A novel 20-membered macrolide, amphidinolide-A, was isolated from the cultured marine dinoflagellate *Amphidinium* sp. It had antitumor activity against murine L1210 leukemia and L5178Y lymphoma cells *in vitro*.

b) Two novel antineoplastic piperidine alkaloids with calmodulin antagonistic activity, pseudodistomins A and B, were isolated from an Okinawan tunicate, *Pseudodistoma kanoko*. They inhibited the growth of murine L1210 and L5178Y cells *in vitro*.

3. Synthesis of novel antitumor compounds.

Seventeen liposoluble *bis* (carboxylato)-cyclohexane-1, 2-diammineplatinum (II) and *bis* (carboxylato)-*cis*-diammineplatinum (II) complexes were synthesized and tested for antitumor activity against leukemia L1210 cells in mice. The former complexes had excellent antitumor activity without any toxicity to the host at the therapeutic dose when used with lipiodol as a carrier solvent. The latter complexes had neither antitumor activity nor toxicity *in vivo*. The former complexes were gradually released from lipiodol to saline *in vitro*; the latter were not. The activity was dependent on the chain length of the carboxylato residue and also on the molecular shape of the ligand part of the complexes. Among them, *bis* (myristato)-cyclohexane-1, 2-diammineplatinum (II) will soon be submitted to Phase I clinical trials.

Furthermore, for development of new, potent anticancer agents derived from naturally occurring and synthetic compounds, we now cooperate with the following researchers: Prof. I. Kitagawa, Osaka University; Prof. H. Kamiya, Kitasato University; Prof. H. Kakizawa, Tsukuba University; Prof. T. Komori, Kyushu University; Dr. N. Fusetani, Tokyo University; Dr. D. Uemura, Shizuoka University; Dr. J. Kobayashi, Mitsubishi-Kasei Institute of Life Sciences; Drs. M. Maeda, N. Takasuka, N.A.Uchida, and H. Uchida, National Cancer Center Research Institute; Prof. T. Ueda, Hokkaido University; Prof. H. Ogura, Kitasato University; Prof. K. Kanematsu, Kyushu University; Prof. Y. Kawazoe, Nagoya City University; Dr. M. Hozumi, Saitama Cancer Center Research Institute; Prof. F. Yoneda, Kyoto University; Prof. K. Shudo, Tokyo University; Prof. S. Kanetomo and Dr. A. Wada, Hokuriku University; Prof. Y. Shimizu, University of Rhode Island; and Prof. B. Wahren, The National Bacteriological Laboratory.

1) School of Fisheries Science, Kitasato University

(2) Development of a new experimental model for prediction of malignancy of tumors and clinical application of the chemosensitivity test with use of embryonated chicks.

T. Sasaki, M. Tanaka, Y. Endo,[1] and Y. Tatsusawa

In the treatment of cancer patients, the schedule of therapy should depend on the degree of malignancy of the tumor, and the selection of suitable anticancer drugs for an individual patient is important. However, there is no established way by which the malignancy of an individual primary cancer and suitable drugs for the patients can be accurately established. Development of such experimental models to predict the malignancy of tumors to evaluate the prognosis for cancer patients and to select a suitable set of anticancer drugs for individual patients could be helpful for clinicians. We have systemically progressed toward the development of such experimental models. Embryonated chicks have been used to graft malignant cells from foreign origins, because they are immune-deficient. We examined the use of embryonated chicks to evaluate the malignancy of tumors and their drug sensitivity for primary and metastatic cancers.

1. Predicting the malignancy of tumors.

We tested metastatic ability as the index of malignancy of tumors, because matastasis is the most important factor to decide the prognosis of the patients. We used chicken chorioallantoic membrane (CAM) to predict the metastatic ability of 3 murine B16 melanoma variants and also of human giant-cell carcinoma of the lung (Lu-65). Tumor cells were deposited onto CAM 10 days after fertilization, and the tumorigenicity of the lungs of the embryo was examined by histologically. Metastases were observed in the lungs both with B16 melanomas and Lu-65. The results accurately reflected the differing matastatic ability of the B16 melanoma variants.

2. Chemosensitivity test of murine transplantable tumors implanted onto CAM.

The CAM of chick embryo was used to examine the chemosensitivity of the murine tumors B16-F1 melanoma, B16-F10 melanoma, Meth-A fibrosarcoma, and Ehrlich carcinoma. The tumors were grown on the membrane, and the effects of 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU), cyclophosphamide (CY), dimethyl-triazeno-immidazole-carboxamide (DTIC), 5-fluorouracil (5-FU), methotrexate (MTX), adriamycin (ADM), mitomycin C (MMC), vincristine (VCR), and cisplatin (CDDP) on the growth of the tumors were tested by injection into the blood vessel of the CAM or into the yolk sac. The use of chick embryos limits the time for drug exposure to 3 or 4 days, but Ehrlich carcinoma and Meth-A fibrosarcoma needed a longer time for the test; the use of irradiation from a cobalt source overcame the problem by increasing the growth rate of the grafts of these tumors. There ap-

peared to be good correlation between the effects of the drugs on B16 melanomas grown in the eggs and in the original animals. Many compounds, including prodrugs such as CY and DTIC, could be assayed in this way.

3. Chemosensitivity test for experimental metastasis with use of B16-F10 melanoma cells.

In the chemosensitivity test for experimental metastasis, B16-F10 cells were injected into the blood vessel on the CAM of 11-day-old chick embryos. The chemosensitivity test was done with ACNU, ADM, CDDP, and CY. As the target organs of metastasis, the brain, lungs, and liver were dissected out and visible tumor nodules on the surface of each organ were counted. Drug sensitivity for the metastasis was measured by comparison of the number of nodules on the organs of the treated group and that of a control group. The results showed slightly different sensitivities for the metastasis of each organ tested. ACNU was effective for the brain only. All drugs tested strongly inhibited lung metastasis at the same dose used for the primary tumor. In particular, CY was the most effective drug for liver metastasis. The prodrug CY requires *in vivo* activation by hepatic microsomal enzymes to become effective. Our results suggested that a large amount of the active form of CY exists in the liver and hence it inhibits implantation and regrowth of melanoma cells in the liver.

4. Chemosensitivity test for primary human cancers.

First of all, we tested how well many primary human cancers obtained by surgery could be grafted to the CAM. Bladder cancer (2 cases), gastrointestinal cancer (3 cases), glioma (3 cases), lung cancer (4 cases), malignant fibrous histiocytoma (9 cases), melanoma (4 cases), osteosarcoma (14 cases), ovarian cancer (7 cases), prostate cancer (9 cases), and uterine cancer (8 cases) were examined, All 63 specimens grew on the CAM (Table I). The tumors on the CAM made solid tumors and necrotic areas were rare. They also induced marked vascularization. These results showed that chick embryos can be used for chemosensitivity tests for primary human cancers.

Chemosensitivity tests for primary human cancers usually use combined chemotherapy. The clinical dose was converted into the injected dose for the chick embryo assays, because the mean body weight of the chick embryos was 5.1g on the day of injection. Twenty-seven cases of ovarian, uterine, and bone and soft tissue cancers were clinically evaluated. The correlation of this assay and the clinical response gave 8 cases of true positive, 13 cases of true negative, and 6 cases of false negative (Table II). The over-all predictive accuracy was 77.8%. These results show that this assay is a promising method to predict the clinical response of primary human cancers.

There is no experimentally useful model of chemosensitivity for human tumor metastasis, even among transplantable animal tumor systems.

Our results showed this assay system with embryonated chicks is an attractive model for the quantitative study of chemosensitivity for primary and metastatic tumors, and also for the development of new antineoplastic drugs including prodrugs, because of its reproducibility, rapidity, sensitivity, convenience, and low cost.

We sincerely thank the following researchers for collaboration in obtaining the correlations between the embryonated chick assay and clinical effects.

Drs. K. Nishikawa, H. Chuman, and H. Fukuma, Dept. of Orthopedic Surgery, National Cancer Center Hospital; Drs. H. Uchida and K. Nitta, Chemotherapy Division, National Cancer Center Research Instisute; Dr. K. Matsumoto, Dept. of Urology, National Cancer Center Hospital; Dr. O. Nakamura, Dept. of Neurosurgery, Tokyo Metropolitan Hospital; Dr. S. Obi, Dept. of Gynecology, Tokyo Senbai Hospital; Dr. K. Ishihara, Dept. of Dermatology, National Cancer Center Hospital; Dr. S. Ikekawa, Dept. of Dermatology, Saitama Medical University; Prof. H. Hisazumi, Drs. T. Uchibayashi, K. Nakajima, T. Asari, and M. Egawa, Dept. of Urology, School of Medicine, Kanazawa University; Prof. M. Mai, and Dr. T. Suga, Dept. of Surgery, Cancer Research Institute, Kanazawa University; Drs. K. Tomita, M. Sugihara, and H. Tsuchiya, Dept. of Orthopedic Surgery, School of Medicine, Kanazawa University; Drs. H. Itoh, K. Shoin, and F. Enkaku, Dept. of Neurosurgery, School of Medicine, Kanazawa University; Dr. Y. Watanabe, 1st Dept. of Surgery, School of Medicine, Kanazawa University; and Dr. Y. Yabuki. Dept. of Gynecology, Ishikawa Prefectural Central Hospital.

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Table I. Growth of Various Human Tumors after Transplant onto the Embryonic Chorioallantoic Membrane

Tumor type	No. of successes/Total of transplants attempted
Bladder cancer	2/2
Gastrointestinal cancer	3/3
Glioma	3/3
Lung cancer	4/4
Malignant fibrous histiocytoma	9/9
Malignant melanoma	4/4
Osteosarcoma	14/14
Ovarian cancer	7/7
Prostate cancer	9/9
Uterine cancer	8/8
Total	63/63

Table II. Correlation between Embryonated Chick Assay and Clinical Effects

Embryonated chick assay/Clinical effect*			
S/S	S/R	R/S	R/R
8	0	6	13

*S/S: True positive
S/R: False positive
R/S: False negative
R/R: True negative

(3) Biosynthesis of interleukin-2 in human peripheral lymphocytes stimulated with OK-432*

S. Koshimura,[1] S.N. Sakai,[2] T. Miyawaki,[3] and H. Satoh[4]

The biosynthesis of interleukin-2 (IL-2) *in vitro* was investigated from the standpoint of immunologic or molecular techniques used in studying human peripheral blood lymphocytes (PBL) stimulated with a streptococcal preparation, OK-432.

The following results were obtained.

1) Two × 10^6 cells of PBL from a healthy donor were cultured with 50μg/ml OK-432 and the production of IL-2 was examined at daily intervals. The production of IL-2 increased exponentially after 48hr, indicating that there were 25 to 30 units in the culture medium. As a control experiment, the same number of PBL was cultured with 0.1% phytohemagglutinin (PHA). The production of IL-2 in the control culture reached a maximum level after 48hr and thereafter decreased rapidly.

2) PBL stimulated with OK-432 did not show a significant proliferative response or expression of the Tac antigen; PBL stimulated with PHA clearly showed both responses.

3) The dose-response pattern with OK-432 in the proliferation of PBL, the expression of the Tac antigen, and the production of interferon-γ and IL-2 were also examined. The proliferative response of PBL was observed in the culture with 20μg/ml OK-432. At this dose, Tac antigen was expressed in about 10% of the stimulated PBL. However, the production of IL-2 was less under these conditions.

4) To demonstrate the production of IL-2 at the translational level, mRNA was purified from PBL stimulated with OK-432 or PHA, and then mRNA that specified IL-2 was detected by the Northern blotting technique, with a ^{32}P-labeled probe used for human IL-2. mRNA bands could be demonstrated human PBL stimulated with either OK-432 or PHA *in vitro* or *in vivo*, but not in PBL cultured *in vitro* without OK-432 or PHA.

*Sakai, S. N., Miyawaki, T., Satoh, H., and Koshimura, S., 6th International Congress of Immunology, Toronto, Canada, 1986

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2) Dept. of Immunobiology, Cancer Research Inst., Kanazawa University.
3) Dept. of Pediatrics, School of Medicine, Kanazawa University.
4) Dept of Virology, Cancer Research Inst., Kanazawa University.

(4) Inhibition of a suppressor cell generation but facilitation of a helper cell appearance by mouse serum in culture.

K. Ryoyama and C. Ryoyama

Normal C57BL/6 mouse spleen cells cultured for 5days in the presence of fetal calf serum (FCS) were shown, in mixing experiments, to suppress the primary humoral response of freshly explanted C57BL/6 spleen cells against SRBC. This suppressor cell generation was largely dependent on the FCS concentration in the suppressor generation culture. Ten or even 5% FCS effectively supported the generation of suppressor cells, but 1% FCS only marginally supported it. When mouse serum (MS) from normal C57BL/6 mice was added to the suppressor generation culture, it inhibited the generation of the suppressor cells. Sera from allogeneic and athymic nude mice were also effective. The active principle(s) of MS was heat-stable (100°C, 30min), not dialyzable, and concentrated into the fraction which was not precipitated by 50% saturation with ammonium sulfate and which was eluted at a concentration of about 0.2M NaCl from a DEAE-Sephadex A-50 column. The inhibition by MS of the suppressor generation was eliminated by an IL2-containing preparation.

Meanwhile, MS did not inhibit the appearance of a helper activity in culture, whereas it inhibited the development of the suppressor cells.

Further investigation of the helper cell developed in cultures supplemented with MS indicated that both *in vivo* and *in vitro* immunizations with SRBC induced the most effective helper activity and SRBC-ghosts or even sonicated SRBC-ghosts were also effective for *in vitro* immunization. An IL 2-containing preparation inhibited the development of a helper activity when added on Day 0 but not on Day 4. rIL2, however, slightly augmented the development of a helper activity even when added on Day 0. The kinetics of the anti-SRBC PFC response did not change in the presence of the helper cell, and augmentation of the response by the helper cell was seen on all days tested. Augmentation occurred without further SRBC addition to anti-SRBC PFC generation cultures whereas it did not occur without "intact" responder spleen cells. The helper cell was resistant to X-irradiation and to treatment with anti-Thy 1. 2 plus C' but sensitive to treatment with anti-Lyt 1. 2 plus C'. The activity of the suppressor cells dominated that of the helper cells, and the helper activity appeared only when the ratio of suppressor cells to helper cells was reduced to less than 1:4.

(5) Enhanced generation of OK-432-induced killer cells by interleukin 2.

T. Ujiie

Spleen cells of untreated mice became cytotoxic in a nonspecific way after being cultured in the presence of a streptococcal preparation, OK-432, for 3 or more days. The toxicity is due to T cells with Lyt-1, 2^- and asialo-GM_1^+ phenotypes. These cells, named OK-432-induced killer (OIK) cells, are cytotoxic to tumor cells resistant to natural killer (NK) cells, but not to YAC-1 cells which are highly vulnerable to NK cells.

Adoptive therapy with syngeneic OIK cells prolongs the survival time of mice bearing the poorly immunogenic EL-4 lymphoma. However, OIK cells are ineffective on inoculation of more than 10^4 lymphoma cells. A method to enhance OIK activity would be of therapeutic use.

C57BL/6 splenic cells cultured for 5 days in the presence of both OK-432 and interleukin 2 (IL-2) were highly cytotoxic to both EL-4 and YAC-1 cells, and had Thy-1^+, Lyt-1, 2^-, and asialo-GM_1^+ phenotypes, which were identical to those of OIK cells. A test competitive inhibition with cold target cells and fractionation by centrifugation onto discontinuous density gradients of Percoll showed that cytotoxic cells generated by OK-432 plus IL-2 comprised at least two populations; the cells in the first population were cytotoxic to EL-4 cells but not to YAC-1 cells, and were smaller in size than those in the second population, which were cytotoxic to YAC-1 cells and had NK morphology. Therefore, generation of OIK cells was augmented by IL-2.

OK-432 augments NK activity *in vitro* and stimulates lymphocytes to produce IL-2 and interferons. These factors are essential for the generation of killer cells. Thus, it seems that native spleen cells responded *in vitro* to such lymphokines, but primarily cultured cells no longer responded to OK-432 and did not produce the lymphokines, although they did respond to IL-2. Macrophage depletion diminished generation of nonspecific killer cells, and so, monokines such as interleukin 1 produced by OK-432-stimulated macrophages might participate in the generation. The primarily cultured cells lacked macrophages, and might respond little to OK-432.

(6) Adoptive therapy of mice bearing tumors with OK-432-induced killer cells.

T. Ujiie

Spleen cells from untreated mice cultured for 3 or more days in a medium with a streptococcal preparation OK-432 became cytotoxic *in vitro* against several allogeneic and syngeneic tumor cells. They are named tentatively OK-432-induced killer (OIK) cells. I report here the *in vivo* antitumor efficacy of OIK cells, manifested by the adoptive immuno- and and immunochemo-therapy of mice bearing syngeneic tumors, such as EL-4 lymphoma, Meth-A fibrosarcoma, and MOPC-31C plasmacytoma, with OIK cells. OIK cells were able to neutralize these tumor cells, as shown by Winn-type tests, and the cell transfer (1×10^7) caused in prolonging the life-spans of mice inoculated intraperitoneally (ip) with 1×10^3 EL-4 or 3×10^5 Meth-A cells. Therapeutic effectiveness of OIK cells was enhanced synergistically by OK-432 (50 KE/kg) plus recombinant interleukin 2 (rIL-2; 50 units/mouse, the gift of Takeda Chemical Industries, Ltd.) concomitantly administered. However, this regimen was ineffective on inoculation of more than 10^4 EL-4 cells. Thus, mice inoculated with 1×10^4 EL-4 cells received ip administration of 200mg/kg cyclophosphamide (CY) on day 3 followed by treatments with OIK cells, OK-432, and rIL-2 6hr later and again on day 6. This chemoimmunotherapy was superior to chemotherapy alone in giving long term survivors. Therefore, the adoptive therapy with nonspecific-type killer cells induced by OK-432 *in vitro* followed by administration of OK-432 and rIL-2 may be available clinically as an adjunct to cytoreductive chemotherapy for cancer.

Table 1. Treatment of mice bearing tumors with CY, OIK cells, OK-432, and rIL-2

	Treatment protocol				Tumors	Antitumor effect	
#	CY	OIK cells	OK-432	rIL-2		AST[a] Mean±SD	S/T[b]
1	No	No	No	No	Meth-A	20.4±1.8	0/5
2	No	Yes	No	No		26.0±2.3	0/5
3	No	No	Yes	No		28.8±4.3	0/5
4	No	No	No	Yes		24.8±1.7	0/5
5	No	No	Yes	Yes		44.0±6.4	0/5
6	No	Yes	Yes	Yes		50.0±0	5/5
7	No	No	No	No	EL-4	13.0±1.0	0/5
8	Yes	No	No	No		20.2±1.3	0/5
9	Yes	Yes	No	No		28.1±2.6	0/5
10	Yes	No	Yes	Yes		20.4±0.6	0/5
11	Yes	Yes	Yes	Yes		43.6±9.2	3/5

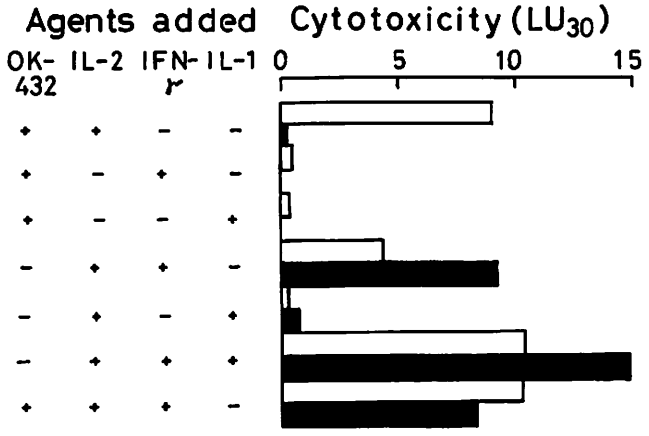
a) Average survival time in days (including 50-day survivors)
b) Fifty-day survivors/tested animals

(7) Generation of activated killer-like cells by OK-432-mediated production of interleukin 1, interleukin 2, and interferon-γ *in vitro*. T. Ujiie

This report is concerned with nonspecific-type-killer cells which were induced from human peripheral blood mononuclear (PBM) cells when they were incubated *in vitro* in the presence of a streptococcal preparation OK-432. The study is centered on their relation to activated killer (AK) cells, which have been defined as activated lymphocytes capable of lysing a variety of target cells, including natural killer (NK)-resistant tumor cell lines and freshly harvested-allogeneic and -autologous solid tumor cells, and which are induced by a variety of culture conditions. AK cells are distinct from classical cytotoxic T lymphocytes (CTL) and NK cells, and are assumed to be similar to or identical to lymphokine-activated killer (LAK) cells.

Antibodies to cytokines, such as anti-interleukin 1 (IL-1) antibody, anti-interleukin 2 (IL-2) antibody, and anti-interferon-γ (IFN-γ) antibody blocked AK-like cell generation by OK-432 *in vitro*, and their generation was potentiated by exogenous lymphokines such as IL-2 and IFN-γ, the former being much more effective than the latter. However, monocyte-depleted PBM cells were barely responsive to OK-432 for inducing the cytotoxic cells. But the monocyte-depleted PBM cells generated the AK cells after being incubated in medium with both IL-2 and IFN-γ. Their generation was augmented by exogenous IL-1 but not by OK-432. These data suggest that the nonspecific type-cytotoxicity induced from human PBM cells by OK-432 *in vitro* needs the participation of macrophage-monocyte lineage cells and mediates primarily the monokine IL-1 production.

Fig. 1 Generation of AK-like cells by IL-1, IL-2, and IFN-γ, produced OK-432 mediation *in vitro*



Unfractionated (open bards, 1×10^6/ml) or monocyte-depleted (closed bards, 0.5×10^6/ml) PBM cells were cultured for 5 days at 37°C in medium containing OK-432 (0.01 KE/ml), IL-2 (10%), IFN-γ (100 units/ml), and IL-1 (1 unit/ml) in various combinations indicated in the figure.

(8) Immunogenic potential of tumor cells after treatment with chemotherapeutic agents.

T. Ujiie

The chemoimmunotherapy of tumors may involve first a reduction in the tumor load by chemotherapeutic drugs, an event accompanied by presentation of tumor antigens. Surviving immunogenic tumor cells may then be removed by immune defense cells, the activity of which can be increased by immunostimulators, such as OK-432. Such a scheme is quite probable, since it has been found that OK-432 strengthens the immunity to tumors induced by attenuated tumor cells, and because animals cured by chemotherapy alone lack resistance to a second challenge with poorly immunogenic tumors. Antigen expression by tumors increases following *in vivo* or *in vitro* treatment with 5-(3,3'-dimethyl-1-triazeno) -imidazole 4-carboxamide, macromomycin, and interferons. Mitomycin C (MMC) prevents the shedding from tumor menbranes of soluble antigens that induce suppressor T cells, thus making such cells useful in the *in vivo* generation of antitumor immunity.

Tumor cells treated with other chemotherapeutic agents such as adriamycin, daunomycin, bleomycin, 4-hydroperoxycyclophosphamide (4-HOOCY, the gift of Shionogi & Co.), ACNU, and *cis*-diamminedichloroplatinum (II) (*cis*-DDP) were evaluated for their ability to induce antitumor immunity *in vitro* and *in vivo*.

Firstly, EL-4 tumor cells were incubated *in vitro* for 30 or 60 min at 37°C with an antitumor agent, and with them splenic cells from syngeneic mice immune or non-immune to EL-4 tumor were cultured at different responder:stimulator cell ratios for 5days *in vitro*. Specific toxicity of cultured spleen cells to EL-4 cells *in vitro* was detected by a ^{51}Cr-release assay. Next, to examine the *in vivo* effect of the tumor cells attenuated by antitumor agents *in vitro*, C57BL/6 mice were sensitized twice with a one-week interval between injections with 1×10^7 attenuated EL-4 cells. OK-432 (100 KE/kg) was given ip the day after each sensitization. The acquired immunity was examined by induced transplantation resistance to challenged EL-4 cells (1×10^3). As the result, EL-4 tumor cells exposed *in vitro* to 4-HOOCY, and to another alkylating agent, *cis*-DDP, as well as MMC, induced development of antitumor immunity in syngeneic mice co-administered with OK-432. The anthracyclines (adriamycin and daunomycin) were very slightly effective. The efficacy of the alkylating agents in inducing tumor immunity was also demonstrated by their ability to stimulate secondary *in vitro* generation of splenic cytotoxicity. These data supported the proposed explanation for effectiveness of chemoimmunotherapy against poorly immunogenic tumors.