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Abstract

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EFFECT OF LYMPHOTOXIN-LIKE SUBSTANCE (OH-1) ON METASTATIC TUMOR PROLIFERATION

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Abstract. The effect of a lymphotoxin-like substance, OH-1, released by human acute lymphatic leukemia BALL-1 cells, on metastatic tumor proliferation was investigated in BDF_1 mice with transplanted Lewis lung carcinoma cells. Mitomycin-C, cyclophosphamide and adriamycin were used as control agents. The effect of OH-1 on metastases, as determined by comparison of the numbers of pulmonary nodules and by ^{3}H-thymidine labeling indices, was significant. Also, investigation of the effect of OH-1 on host immunity showed that, while the control preparations had considerable side effects, immunodepression and emaciation were not noted with OH-1. As to direct cytotoxicity, OH-1 is principally cytostatic in activity and effects cell progression delay in both the G_1 and G_2 phases.

Key words : lung metastasis, cell cycle, autoradiography, lymphotoxin, NK activity.

The farthest possible surgical excision, the least possible tumor residue and immuno-chemo therapy are remedies generally accepted today for the solid cancers we surgeons treat (1). Since, however, the cellular immunity of cancer patients declines after surgery (2, 3), administration of an anti-tumor agent able to exert a negative effect on the tumor-host relation presents an issue from the standpoint of immunology.

Numerous lymphokines have been reported since Dumonde's report (4). Lymphokines can be classified into two groups: those which activate effector cells, such as K-cells, NK cells and cytotoxic T-cells, while indirectly inhibiting tumor cells, and those which directly inhibit tumor cells. The tumor necrosis factor (TNF) and lymphotoxins are well known members of the latter group. Presumably, OH-1 is a member of the latter group. We transplanted mice with Lewis lung carcinoma cells, an experimental model which readily metastasizes postoperatively, and investigated OH-1 as to its metastic inhibitory effect. Additionally, the effect of OH-1 on the tumor-host relationship was compared with those of other anti-cancer agents.

MATERIALS AND METHODS

Experimental animals. Eight-week-old female BDF_1 and DDS mice, about 25 g each, were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan, and used in the experiments.

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Tumors. Lewis lung carcinoma cells (3LL): Cells successively maintained subcutaneously in C57BL/6 mice by The First Department of Surgery, Okayama University Medical School were used. For the experiment, the tumor was excised aseptically 10 days after the successive transplantation, minced, washed three times in Hanks solution, treated with 0.25 % trypsin (Difco Co., U.S.A.) at 37℃ for 15 min, washed twice with Eagle's essential medium supplemented with 10 % FCS, filtered through #80 and #150 wire meshes and prepared into a single cell suspension. Ehrlich ascites carcinoma cells: Cells successively maintained in the peritoneal cavity of DDS mice at The First Department of Surgery, Okayama University Medical School were used. In the experiment, the tumor cells obtained from mice ascites 7 days after the successive transplantation were washed three times in Hanks solution.

Lymphotoxin-like substance (OH-1). A lymphotoxin-like substance, OH-1, supplied by Hayashibara Biochemical Laboratories, Inc., Okayama, was used. Firstly, newborn hamsters were subcutaneously transplanted within 24 h of birth with human lymphatic leukemic BALL-1 cells. The hamsters were injected with rabbit anti-hamster thymocyte serum twice a week to allow proliferation of the cells. The grown tumor mass was excised 3-4 weeks after the transplantation, minced and filtered to obtain a cell suspension. The resultant cells were adjusted to 5×10^6 cells/ml with serum-free RPMI 1640 medium, placed in a spinner flask, mixed with Sendai virus to bring the concentration to 100 HA units/ml and then cultured at pH 7.2 and 37℃ for 20 h. The supernatant thus obtained was salted out with ammonium sulphate, and dialyzed against phosphate buffer to obtain a concentrated protein solution. The resultant solution was applied on an anti-human IFN-α bond Sepharose column to remove the human IFN-α produced by the viral induction. A fraction with a molecular weight of 10,000 - 100,000 was obtained by gelchromatography after repeated salting-out and dialysis. The fraction was designated as OH-1. Its cytotoxicity was determined by the highly sensitive and rapid assay of Eifel *et al.* (5) for lymphotoxin, using mice L929 cells as the target cells. One unit of activity is defined as the reciprocal of the dilution that produces a cytopathic effect (CPE) in 50 % of the target cells.

Control anti-cancer agents. Mitomycin-C (MMC) and adriamycin (ADM), both of Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan, and cyclophosphamide (CY), Shionogi & Co., Ltd., Osaka, Japan, were used as controls.

Determination of lung metastasis. BDF_1 mice were transplanted in their foot pad with 1×10^6 3LL cells. Their primary tumor was removed by femoral amputation under ether anesthesia on the 10th day from the transplantation, and their lung metastatic nodules were evaluated on the 21st day from the transplantation. The evaluation was carried out by the Wexler method (6). Separately, mice treated under the same conditions were administered intraperitoneally on the 21st day with 10 μCi/g body weight of [methyl-^{3}H] thymidine (TRK, 120, specific activity 25 Ci/m mol, *Japan Isotope Association*) and sacrificed 60 min later by decapitation. Their lungs were excised in one piece. After fixation in 10 % buffered formalin and embedding in paraffin, the masses were prepared into continuous slices of 3-4 μm, and then the ^{3}H-TdR indices of lung metastases wrer investigated by autoradiography.

Cell cycle determination. DDS mice were injected intraperitoneally with 1×10^6 Ehrlich ascites carcinoma cells, and the mice were divided into two groups of two mice with similar ascites pools on the 10th day (7, 8). The mice were administered saline (control) or 200,000 u/kg OH-1 into their intraperitoneal cavities, and thereafter twice with 5 μCi/g [methyl-^{3}H] thymidine 30 min and 9.5 h later. Ascites samples collected successively from the first [methyl-^{3}H] thymidine administration were prepared into fixed smears. The smears were subjected to autoradiography, and the labeled mitotic indices and thymidine labeling indices were investi-

gated and expressed in percentages.

Autoradiography. Autoradiography was performed according to the method of Fujita and Kitamura (9). Slide preparations were immersed in Sakura Emulsion NR-M_2, a product of Konishiroku Photo Industry Co., Ltd., Tokyo, Japan, dried at room temperature, and placed in a camera together with silica gel. The slides were exposed at 4℃ for four weeks, immersed in an 18℃ developing solution, Konidol X, Konishiroku Photo, for five min and then in a stop solution, followed by immediate immersion in a fixing solution of Konifix, Konishiroku Photo, for 15 min. The plates were dried at room temperature, stained with hematoxylin and eosin, and subjected to microscopic scoring (10×40) to determine thymidine labeling indices and mitotic indices. Labeled cells were identified by the darkened tight aggregates of silver particles.

Determination of spleen cell NK activity. BDF_1 mice were transplanted in one of their foot pads with 1×10^6 3LL cells, and their legs bearing the tumor were excised on the tenth day. From the following day, the mice were iv injected daily with saline, 50,000 u/kg OH-1, 25 mg/kg CY or 0.5 mg/kg MMC. On the 3rd, 7th and 10th days from the injection, spleens were excised from the mice, minced and filtered through #150 wire mesh. After washing, the spleen cells were used as effector cells. YAC-1 cells labeled with ^{51}Cr were used as target cells, and mixed with spleen cells in Microtest II plates to give an E/T ratio of 200:1. After six hours of incubation, the supernatant was subjected to γ-spectrometer (Fig. 1). The following equation was used to determine % cytolysis:

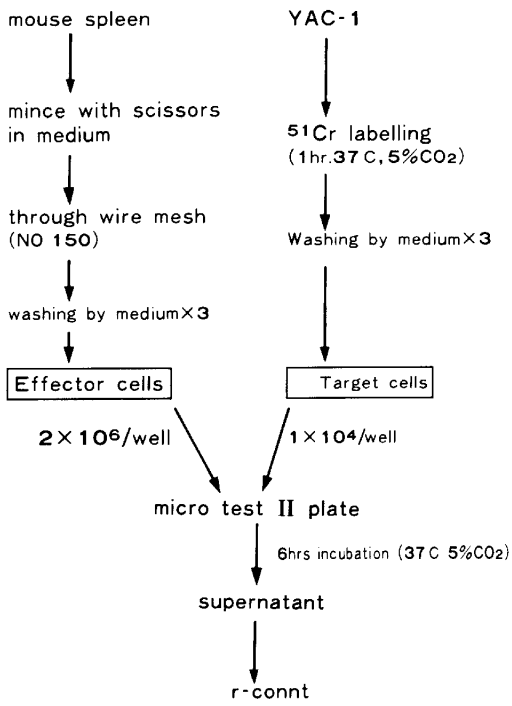

Fig. 1. Determination of spleen cell NK activity.

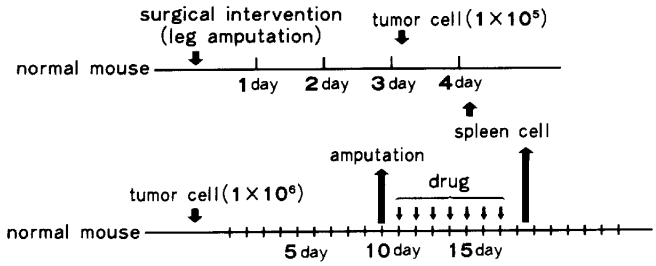

Fig. 2. Determination of concomitant immunity.

$$\text{cytolysis (\%)} = \frac{\text{mean experimental cpm} - \text{mean spontaneous cpm}}{\text{mean total cpm} - \text{mean spontaneous cpm}} \times 100$$

Determination of concomitant immunity. One leg of BDF_1 mice was excised under ether anesthesia, 1×10^5 3LL cells were transplanted in their tail veins three days after the excision, and 2×10^7 cells of the spleen cell preparation were intravenously administered 24 h later. The spleen cells used for the intravenous administration were collected from mice transplanted with 1×10^6 3LL into their foot pad. One leg of each of these mice was amputated on the tenth day, and the mice were administered from the following day for seven days with saline, 50,000 u/kg/day OH-1, 25 mg/kg/day CY, or 0.5 mg/kg/day MMC. Then the lung metastatic nodules were counted on the 21st day from the tumor transplantation (Fig. 2).

RESULTS

The Effect of OH-1 on Lung Metastases and Survival Rate

The effect on lung metastatic nodules. After excision of primary tumors on the 10th day after the tumor transplantation, the mice were administered into their tail veins successively for ten days with saline, 50,000 u/kg/day OH-1, 25 mg/kg/day CY, 0.5 mg/kg/day MMC or 2.5 mg/kg/day ADM. On the 21st day, the lung metastatic nodules were counted. The average number of lung metastatic nodules was for the saline group 42.0; the OH-1 group 6.2; the CY group 3.0; the MMC group 34.0 and the ADM group 20.8, showing that a significant efficacy is attained with OH-1 or CY (Table 1).

TABLE 1. THE EFFECT OF OH-1 ON LUNG METASTATIC NODULES

	Pulmonary nodules (mean ± SE)	Pulmonary metastasis		p (t-test)
		Range	Incidence	
Saline	42.0 ± 7.8	31 – 50	7/7	
OH-1 (50,000 u/kg/day)	6.2 ± 4.3	0 – 12	6/7	0.001
Cyclophosphamide 25 mg/kg/day)	3.0 ± 2.2	0 – 5	6/7	0.001
Mitomycin-C (0.5 mg/kg/day)	34.0 ± 17.3	31 – 50	7/7	NS
Adriamycin (2.5 mg/kg/day)	20.8 ± 6.1	11 – 27	7/7	0.01

Effect on labeling index of lung metastatic lesions. Mice treated similarly as above were administered intraperitoneally with [methyl-^{3}H[thymidine, and their labeling indices in percentage were checked. The results were 47 % for the saline group, 26 % for OH-1, 23.5 % for CY, 44 % for MMC and 41 % for ADM. The levels were in close correlation with lung metastatic nodules (Fig. 3).

Effect on survival time. The average survival times of the mice treated similarly as above were; saline group, 25.75 ± 1.58 days (mean ± SD); OH-1 group, 31.75 ± 5.28; CY group, 27.00 ± 2.45; MMC group, 24.25 ± 0.71; and ADM group, 26.63 ± 1.51. Only the OH-1 group showed a significantly prolonged survival time over the saline group ($p<0.01$, Fig. 4). In addition, significant emaciation was noted in the CY- and MMC-administered groups.

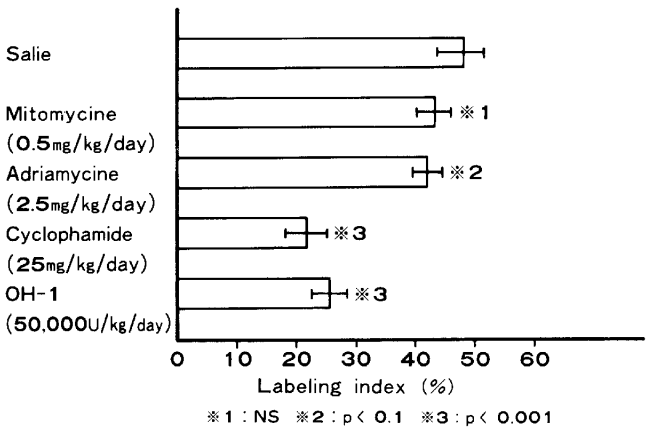

Fig. 3. Effect of OH-1 on labeling index of lung metastatic lesion.

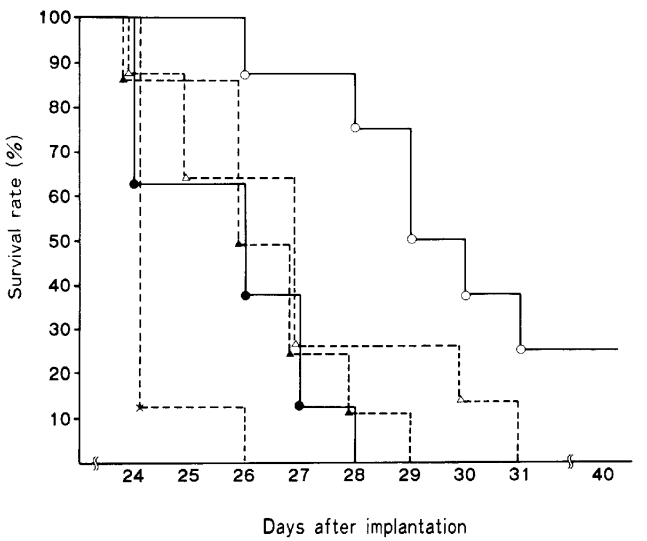

Fig. 4. Effect of OH-1 on survival time. BDF_1 mice were transplanted in one of their foot pads with 1×10^6 3LL cells, and their legs bearing the tumor were excised on the 10th day. From the following day the mice were iv injected successively with a daily dose of saline (●——●), 50,000 u/kg OH-1 (○——○), 25 mg/kg CY(△——△), 0.5 mg/kg MMC (×——×) or 2.5 mg/kg adriamycin (▲——▲).

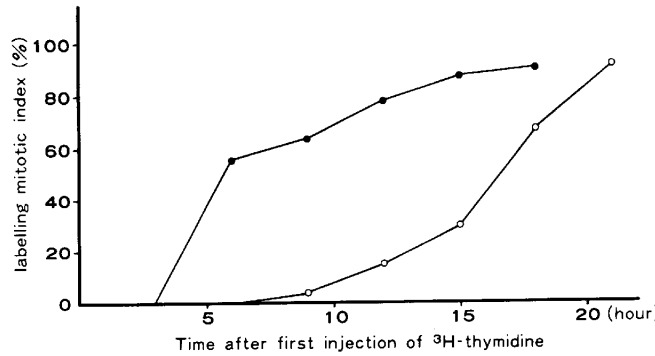

Fig. 5. Effect of OH-1 on cell cycle. DDS mice were injected intraperitoneally with 1×10^6 Ehrlich ascites carcinoma cells, and the mice were divided into two groups of two mice each with similar ascites pools on the 10th day. The mice were administered into their intraperitoneal cavities saline (●——●), or 200,000 u/kg OH-1 (○——○), and thereafter twice with 5 μCi/g [methyl-^{3}H] thymidine 30 min and 9.5 h later.

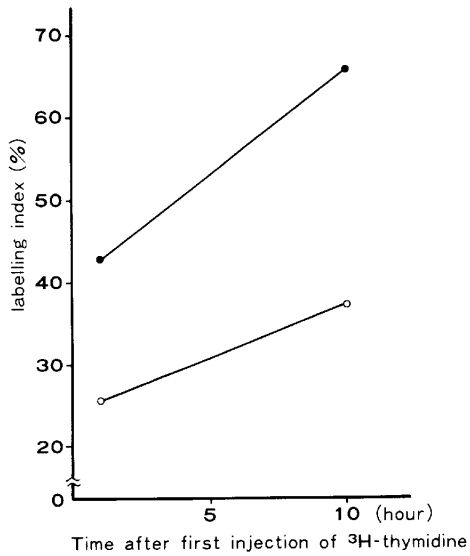

Fig. 6. Effect of OH-1 on cell cycle.

Effect of OH-1 on cell cycle. The mitotic index and labeling index were progressively investigated with Ehrlich-ascites-carcinoma-bearing DDS mice. The progressive change in mitotic indices which presumably reflects the G_2 phase was as shown in Fig. 5. The G_2 phase, defined as the period up to when the mitotic index percentage attains 50, was for the saline group, 6 h and for the OH-1 group, 16.5 h. Fig. 6 shows the results of the investigation of the increase in the labeling

index after two doses of [methyl-^{3}H] thymidine after administration of the specimens. Since the increase in the labeling index should also be inhibited along with the inhibition of migration from the G_1 phase to the S phase, the increased rate presumably reflects action on the G_1 phase. The results showed that a higher inhibition of labeling index increase, relative to that of the saline group, was attained in the OH-1 group. Based on the above findings, OH-1 presumably effects cell progression delay in both the G_1 and G_2 phases.

The effect of OH-1 on the spleen cell NK activity. The primary lesion was excised on the tenth day from tumor transplantation, and one of the agents was administered intraperitoneally and daily. On the 3rd, 7th and 10th days from the starting day of the administration, the spleen NK activities were as shown in Fig. 7. While on the seventh day the saline group gave an NK activity of 25.9 % and the CY and MMC groups showed significant NK activity declines to 13.2 % and 6.6 %, respectively, the OH-1 group showed no decline in NK activity.

Effect of OH-1 on concomitant tumor immunity. Whereas the non-treated control group had an average of 13.43 ± 6.00 (mean ± SD) lung nodules after iv transplantation of 3LL cells, the number for the group to which spleen cells from normal mice were transplanted 24 h after the iv transplantation was 12.57 ± 4.35. The spleen cells of normal mice showed no favorable effect on artificial metastasis. Treatment with spleen cells, derived from mice from which the primary tumor lesion was removed and which were treated with saline, decreased the number of lung nodules to 5.00 ± 2.89 ($p<0.01$). The results show that the spleen cells from tumor-bearing mice have concomitant immunity against the tumor. The

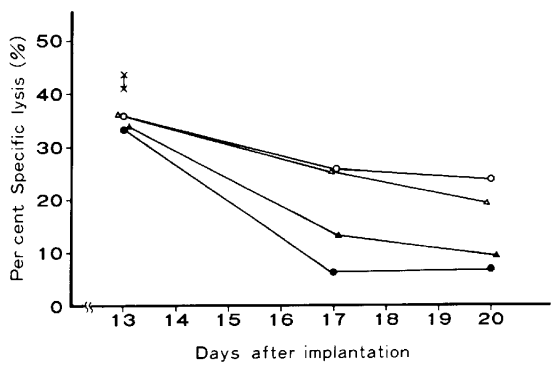

Fig. 7. The Effect of OH-1 on the spleen cell NK activity. BDF_1 mice were transplanted in one of their foot pads with 1×10^6 3LL cells, and their legs bearing the tumor were excised on the 10th day. From the following day the mice were iv injected successively with a daily dose of saline (△——△), 50,000 u/kg OH-1 (○——○), 25 mg/kg CY (▲——▲), or 0.5 mg/kg MMC (●——●). Results obtained with mice neither receiving a transplant nor being treated are plotted (×——×). On the 3rd, 7th and 10th day from the initial injection, spleens were excised from the mice and used as effector cells. YAC-1 cells were used as target cells, and mixed with spleen cells to give an E/T ratio of 200:1.

number of lung nodules of the group injected with spleen cells and administered with MMC or CY after the primary tumor was removed was 14.29 ± 4.39 and 13.14 ± 3.90, respectively, indicating that the administration of MMC or CY effected the disappearance of the concomitant immunity of spleen cells. The number of lung nodules of the mice group injected with spleen cells from the OH-1 administered group was 4.00 ± 1.63, presumably showing that concomitant immunity does not disappear with OH-1 administration (Table 2).

TABLE 2. EFFECT OF OH-1 ON CONCOMITANT TUMOR IMMUNITY

Treatment	Pulmonary nodules	Pulmonary metastasis		p (t-test)
		Range	Incidence	
Control (1)	13.43 ± 6.00	7 – 25	7/7	
Healthy (2)	12.57 ± 4.35	9 – 20	7/7	
Saline (3)	5.00 ± 2.89	2 – 9	7/7	<0.01
Mitomycin (4)	14.29 ± 4.39	9 – 20	7/7	
Cyclophosphamide (5)	13.14 ± 3.90	7 – 18	7/7	
OH-1 (6)	4.00 ± 1.63	2 – 7	7/7	<0.005

*Pulmonary metastases were obtained from leg-amputated mice which were iv transplanted with 1×10^5 3LL cells (control (1)) and, after 24 h, iv injected with 1×10^7 spleen cells recovered from different mice transplanted with 1×10^6 cells, amputated and treated for 7 days with saline (3), MMC (4), CY (5) or OH-1 (6). The controls were untreated mice (2).

DISCUSSION

There is no doubt that surgery is the most effective treatment for solid tumors. Today, the fact that a number of cancer patients are operated at too late a stage because of the incredible depravation of the tumor or because of metastasis is pitiful but true. Elucidation of the metastatic mechanism has been attempted widely around the world. The relationship between the primary tumor and metastases is an interesting and a rewardable subject for investigation from the standpoint of postoperative treatment. It is well known that the tumor grows in accordance with the Gömpertz function, and also that the tumor-doubling time shortens as the tumor mass is removed by surgery or other means. Presumably, the shortening of the length of the mean cell cycle of the proliferating tumor cells and the increase of proliferating cell populations are the causes of the phenomena. The shortening of the length of the cell cycle was experimentally demonstrated by Schabel *et al.* (10), and in human ovarian cancer by Sheehy (11). Simpson-Herren *et al.* observed an increase in proliferated fractions in C57BL/6 mice with Lewis lung carcinoma (12-14). More particularly, they investigated the incorporation of ^{3}H-thymidine into lung metastases by autoradiography, and compared the progressive variation of the labeling index (LI) with those of primary-tumor excised mice and non-excised mice. Based on the results, the investigators claimed that LI, the

index of the proliferated cell population, decreased progressively in the primary-tumor bearing mice whereas the primary-tumor excised mice maintained a high level. Hashimoto (15) reported similar results in their experiment performed similary. The promotion of metastasis by primary-tumor excision is frequently observed in experimental animals (16-18) and also in clinical trials (19). The phenomenon was investigated and reported in detail by E. Gorelik *et al.* (20-23). The facts that metastatic promotion does not disappear on primary-tumor excision in mice whose immunity was inhibited by irradiation and cyclophosphamide administration, that in beige and nude mice which have "concomitant immunity", a rejection of re-transplantation of a secondary tumor occurs in the presence of the tumor, and that splenectomy has no influence on the metastatic promotion phenomenon which is observed after primary-tumor excision, suggest the presence of an directly inhibiting factor of the primary tumor other than that mediated by the immune system. The effect of systematic anesthesia was investigated, but gave similar results. A mechanism which inhibits tumor growth, probably a humoral factor because it effects remote metastases, may exist in the tumor cell itself. As described above, the metastatic lesion left after excision of the primary tumor, presumably the main culprit, may become a favorite target cell from the chemotherapeutic standpoint because many anti-cancer agents show an intensive sensitivity to the proliferative fraction of a tumor. Thus, to perform a reasonable therapy, understanding of the parameters of the inhibition of the cell cycle process by anti-cancer agents is important in choosing the proper agents for combined use, and in determining the appropriate administration interval and timing. The OH-1 specimen we tested acted effectively on lung metastasis after primary tumor excision. In the cell cycle experiment, the substance produced cell progression delays in the G_1 and G_2 phases. Since *in vitro* preliminary ^{51}Cr release tests and ^{3}H-thymidine uptake tests for human cancer cell lines indicated that OH-1 predominantly inhibits ^{3}H-thymidine uptake (unpublished data), OH-1 is likely a substance with cytostatic activity, *i.e.* OH-1 acts cytostatically and effects cell progression delays in the G_1 and G_2 phases, eventually causing cellular accumulation in the G_1 and G_2 phases. According to Terashima's claim (24) on tumor therapy, the combined use of an agent cytocidally sensitive to cells in the G_1 and G_2 phases may further augment the efficacy of OH-1.

In chemotherapy for metastases, the effect of the anti-cancer agent on the host response to tumors presents an issue that should not be ignored. The host sensibility to metastases has been especially covered from an immunological standpoint. As demonstrated by the investigation by Davey *et al.* with mouse lymphoma (25) and that by Eccks with nude rats (26) the participation of T-cells is of course possible. However, the immuno cell lines most discussed lately are macrophages and NK cells. Jones *et al.* (27) showed that metastasis in cases of Lewis lung carcinoma is inhibited by the administration of *Corynebacterium parvum* and proceeds with the administration of macrophage toxins such as silica and cortisone, and

Kagawa *et al.* (28) observed that celiac macrophages activated with N-CWS inhibited metastasis of Lewis lung carcinoma. Talmadge *et al.* (29, 30) administered tumor cells, sensitive or non-sensitive to NK cells, to beige mice and found that NK cells play an important role in metastasis. Hanna (31) reported that low NK activity sensitive young mice and cyclophosphamide-treated mice have considerably higher incidences of lung metastasis, and that the injection of NK cells inhibits metastatic promotion. As described above, the host immunity system plays an important role in metastasis. Mantovani *et al.* (32) investigated in C57BL/6J mice the effects of azathioprine (AZA), cyclophosphamide (CY), dimethyltriazenoimidazol carboxamide (DTIC) and adriamycin (AM) on NK activity. According to their results, while CY inhibits NK activity dose-dependently, AM does not inhibit NK activity per cell but reduces the number of cells. Saijo *et al.* (33) reported their test on the change in NK activity following chemotherapy of 15 lung cancer patients. The investigators used different anti-cancer agents, but in all of the 15 patients, NK activity started declining after two weeks from the first administration, and the activity attained a minimum within three to four weeks. In fact, van Putten (34), Steel (35), Richard (36) and Marie-France (37) reported that lung metastasis was promoted by anti-cancer agents in animal experiments.

Although the anti-cancer agents used in the present experiment showed efficacy on lung metastasis, the administration of them was unable to prolong the mean survival rate, and the NK activity showed a remarkable decline on the 7th day from the start of the administration. Also in the spleen cell transplantation test, the anti-cancer agent eliminated the resistance of spleen cells against the tumor, a property which spleen cells obtain in the presence of tumors. The resistance was also demonstrated by Luca Milas *et al.* (38). The optimal protocol and dosage for the anti-cancer agents used in the present study require elucidation. In cancer therapy, if an anti-cancer agent lacks sufficient specificity to cancer cells, the agent will possibly exert a negative effect on the body. On the contrary, OH-1 exerts its efficacies sufficiently on lung metastasis, and also on survival period prolongation. OH-1, however, has no adverse effect on NK activity as well as on the resistance of spleen cells to the tumor. It may be possible to safely administer OH-1 to postoperative patients with a reduced immunity. Lymphotoxin was introduced by Ruddle *et al.* in 1967 (39) as a cytotoxic factor found in the supernatant of lymphocyte culture challenged with antigen or mitogen, and was named by Granger in 1968 (40). Reportedly, the molecular weight ranges from 12,000 to 120,000 (41-43). The factors existing *in vivo* are presumed mostly to be α- and β-types with a molecular weight in the range of 35,000 - 90,000. Today many reports are available on *in vitro* tests demonstrating the cytotoxicity of lymphotoxins (44-46). Evans (47) reported that lymphotoxin activity is more sensitive in tumor cells than in normal cells. Recently an experiment showing that lymphotoxin inhibits the cariogenesis of cells induced by carcinogen (48), and another showing that lymphotoxin augments the sensitivity of cells to NK activity (49) were reported. Lymphotoxin is

gradually drawing much attention and expectation as a promising anti-tumor agent.

OH-1 is a substance induced by lymphatic cells, and its molecular weight is presumed to be 10,000 - 100,000. Because the substance exhibits a direct cytotoxicity, it is regarded as a lymphotoxin-like substance. Extraction of OH-1 in pure form has not been successful. We took special consideration of the possible complex activity of the various possible contaminants in the supernatant in carrying out our investigation on the physiological properties of OH-1. Nonetheless, mass production of OH-1 is feasible with the utilization of hamsters. Further possible variations or modifications of the substance by replacing the inducer, HVJ, with antigen or mitogen may open new avenues to extract and obtain many more promising and powerful substances of clinical importance and efficacy.

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