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Enhancement of Antitumor Activity in Mouse Alveolar Macrophages by Immunoactivators Encapsulated within Polysaccharide-coated Liposomes

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Abbreviations used in this paper : AM, alveolar macrophages ; CRPMI, RPMI1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin G and streptomycin ; MDP, muramyl dipeptide ; MDP-L18, N-acetylmuramyl-L-alanyl-L-isoglutamine ; MA-CDA, poly (maleic anhydride-alt-2-cyclohexyl-1, 3-dioxep-5-ene) ; PBS, phosphate-buffered saline ; LUV, large unilamellar liposomes ; MLV, multilamellar liposomes ; CHM-LUV, cholesterol-mannan-coated LUV ; CHM-MLV, cholesterol-mannan-coated MLV ; CHM-LUV (MDP-L18), CHM-LUV containing MDP-L18 ; CHM-LUV (MA-CDA), CHM-LUV containing MA-CDA.

ABSTRACT : For active cancer immunotherapy by alveolar macrophage (AM) targeting, the usefulness of polysaccharide-coated liposomes was examined in an animal model, and the morphological changes in activated AM were observed using scanning electron microscopy. Among the liposomes tested, cholesterol-mannan-coated large unilamellar liposomes (CHM-LUV) had the highest targetability to the lung. MDP-L18 or MA-CDA as synthetic immunoactivators, were encapsulated within CHM-LUV. After the intravenous injection of encapsulated or nonencapsulated, MA-CDA or MDP-L18, the antitumor activity of C57BL/6 AM against Lewis lung carcinoma (3LL) was measured in vitro. The encapsulated immunoactivators enhanced the antitumor activity of AM more effectively and for a longer time than the nonencapsulated ones. Concurrent with activation by MA-CDA, AM showed signs of very high surface activity, such as prominent ruffles, elongated filopodia and thickened lamellipodia. Some of the AM showed strong attachment to 3LL cells in vitro. No prolonged survival was obtained with the treatment of 3LL-bearing mice by CHM-LUV (MA-CDA) alone. However, these date demonstrated that polysaccharide-coated liposomes containing immunoactivator might be useful in future active cancer immunotherapy by AM targeting.

INTRODUCTION

Immune cells, such as macrophages, killer T-cells and natural killer cells, play important roles in cancer treatment. Active immunotherapy procedures that enhance the antitumor activity of these cells have been studied. For

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effective immunotherapy procedures, many investigators have developed immunoactivators which can specifically activate these cells while leaving the host unharmed, and methods of their administration have been studied (1).

Liposomes as convenient drug carriers in the treatment of cancer and infectious diseases have been investigated in many laboratories (2, 3), and a few clinical applications of liposomes have been reported (4, 5). In the treatment of cancer, antitumor agents or immunoactivators have been encapsulated in liposomes, and successful results have been reported in animal models (6−9). SUNAMOTO *et al.* developed polysaccharide-coated liposomes, which were more stable against external stimuli in blood than conventional liposomes and had specific organ targetability (10, 11).

In the present study, the usefulness of polysaccharide-coated liposomes for active cancer immunotherapy by alveolar macrophages (AM) targeting was examined, and the surface activity of activated AM using scanning electron microscopy was also observed.

MATERIALS AND METHODS

Animals. Specific-pathogen-free male C57 BL/6 mice, five to seven weeks old, were obtained from Charles River Japan Inc. Mice were fed in a clean house at Nagasaki University Laboratory Animal Center.

Cell Cultures and Reagents. The syngeneic tumor, Lewis lung carcinoma (3LL), was a gift from Chugai Pharmaceutical Co., Japan, and maintained in CRPMI. The AM-mediated cytotoxicity and cytostasis assay were always carried out with 3LL in the exponential growth phase. All cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2 in CRPMI.

MDP-L18, a MDP derivative, was a gift from Daiichi Pharmaceutical Co., Japan. MA-CDA, a synthetic polyanion, was a gift from Raphael M. OTTENBRITE (Virginia Commonwealth University, Richmond, Virginia, U.S.A.), who has synthesized a large number of polyanions and examined their antitumor activities (12). Among these synthetic polyanions, MA-CDA has notably high antitumor activity ; its structure is shown in Fig. 1.

MA−CDA=poly (maleic acid−alt−
2−cyclohexyl−1, 3−dioxep−5−ene)

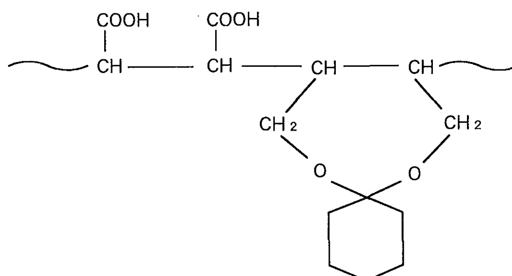

Fig. 1. The structure of MA-CDA.

Preparation of AM. AM were harvested by a tracheobronchial lavage method (13). Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium, and swabbed with iodine in an airflow hood. After opening the peritoneal cavity, mice were exsanguinated by cardiac puncture. The trachea was cannulated with an intravenous catheter (ATOM, Inc., Tokyo) after thoracotomy. The lungs were lavaged with 0.5-0.7 ml of Ca^{2+}- and Mg^{2+}- free PBS at 4℃, and five to seven ml of lavage fluid was recovered per mouse. Five lavage fluid samples were pooled and washed twice by centrifugation at 4℃ for 10 min at 260g ; the cell pellet was resuspended in CRPMI. Then, viability counts and characterization of AM were performed. The harvested cells were found to be more than 95% viable by trypan blue dye exclusion, and composed of more than 96% AM on the basis of nonspecific esterase staining and morphology. Then, 2×10^5 or 1×10^5 harvested cells were plated in each well of a Falcon microtiter plate (No. 3040, Oxnard, Calif.). After a 60-min incubation, plated cells were gently washed twice by filling the wells with CRPMI to eliminate nonadherent cells containing neutrophils and small mononuclear cells. The mean number of nonadherent cells per well was less than 10% of initial plated cells in 10 experiments.

Preparation of Liposomes. CHM-coated liposomes, as one type of polysaccharide-coated liposomes, were prepared by the method

reported by Junzo Sunamoto (10, 11). In addition, LUV containing MA-CDA in the interior core were prepared by the same procedure as that reported by Szoka, *et al* (14, 15). In order to study in vivo uptake of liposomes by AM, MLV were labeled with a lipophilic fluorescent probe (terbium triacetylacetate, Tb $(acac)_3$) which was donated by L. Varallino (Virginia Commonwealth University, Richmond, Virinia, U.S.A.).

In Vivo Uptake of Liposomes by AM. The cells (AM>96%), harvested by lavage 1 or 24 h after intravenous injection of terbium-labeled CHM-MLV, were resuspended in PBS containing 10% FCS, and cytocentrifuge smears stained with May-Giemsa's stain were prepared. Fluorescence of AM was then observed by fluorescence microscopy with ultraviolet excitation, and photographed.

In Vivo Activation of AM by Immunoactivators (MDP-L18 or MA-CDA). Mice were injected intravenously with either nonencapsulated immunoactivator, or immunoactivator or PBS encapsulated within CHM-LUV. A control group of mice were injected with PBS. The CHM-LUV were suspended in a volume of 0.2 ml PBS. AM were harvested at various times after injection, and their tumor cytotoxic properties were assayed in vitro as follows.

The statistically significant differences between groups was determined by paired Student's t test.

AM-mediated Cytotoxicity Assay. AM-mediated cytotoxicity was measured in an 18h ^{51}Cr release assay. Briefly, samples of 10^4 target cells (3LL) prelabeled with $[^{51}Cr]$ Na_2CrO_4 (lmCi/ml) for one hour were added to triplicate wells containing 1×10^5 AM or to empty control wells. The mixtures were incubated at 37℃ for 18h, and then radioactivities of aliquots of the supernatant were counted in a gamma-counter. Maximum release was determined by lysis of the tumor cells with 1N HCl. The cytotoxic activity of AM was calculated using the following formula : % cytotoxicity

$$= \frac{\text{cpm(activated AM+3LL)} - \text{cpm(untreated AM+3LL)}}{\text{cpm(maximum release)} - \text{cpm(untreated AM+3LL)}} \times 100$$

AM-mediated Cytostasis Assay. After the preparation of AM monolayers described above, target cells (3LL) were added to triplicate wells containing AM or to empty control wells. Plates were incubated for 48 h, and 0.1 μCi $[^3H]$ thymidine (15.1 Ci/mmol ; New England Nuclear, Boston, Mass., U.S.A.) was added to the wells 16 h before culture completion.

After 48-h incubation, cells were harvested on a glass filter using a Labo Mash multiple cell harvester (Labo Science Co., Tokyo). The radioactivity of $[^3H]$ thymidine incorporated into 3LL was measured by liquid scintillation. The cytostatic activity of AM was calculated using the following formula :

$$\%\ \text{cytostasis} = \frac{\text{cpm(3LL alone)} - \text{cpm(AM+3LL)}}{\text{cpm(3LL alone)}} \times 100$$

Preparation of Specimens for Scanning Electron Microscopic Examination. AM (1×10^5/well) were plated onto 5-mm-diameter glass coverslips placed in wells of a Microtest III plate. In order to study the interaction between AM and target cells, 1×10^4 3LL were added to AM, incubated for 24 h, and vigorously washed twice with CRPMI. The coverslips were fixed in 1% glutaraldehyde for one hour, rinsed twice with PBS, and postfixed in 1% osmium tetroxide at 4℃ for 1 h. The samples were dehydrated with a graded series of ethanol and isoamylacetate. They were then critical-point dried in a Polaron E 3000 criticalpoint drier, coated with gold-palladium, and examined with a JEOL JEM-100CX electron microscope (JEOL Ltd., Japan) at 10 kV.

Trial in Treatment of Tumor-Bearing Mice by CHM-LUV(MA-CDA). C57BL/6 mice were injected subcutaneously in the footpad with 1×10^5 viable 3LL tumor cells suspended in 0.05 ml of PBS. Approximately two weeks later, when the tumors reached about 10 mm in diameter, microscopic pulmonary metastases were observed in most of the mice (data not shown).

CHM-LUV (MA-CDA) treatment was done at five, eight, and 11 days after the inoculation of tumor to inhibit pulmonary metastasis. At 12 days post-inoculation, the mice were anesthetized by pentobarbital sodium and the tumor-bearing leg (primary tumor) was amputated at midfemur. Treatment of liposomes consisted of a tail vein injection of CHM-LUV suspended in 0.2 ml of PBS. The CHM-LUV contained either MA-CDA (1000 μg per mouse) or PBS. Additional controls included mice injected intravenousely with tree MA-CDA (1000 μg per mouse) or PBS.

Survival rate in each treatment groups was calculated according to the method of Kaplan and Meier (16) .

RESULTS

Uptake of MLV by AM. AM harvested by pulmonary lavage were examined by fluorescent microscopy to determine whether AM had phagocytized CHM-MLV circulating in the peripheral blood. AM were harvested one or 24 h after intravenous injection of terbium-labeled CHM-MLV. The labeled CHM-MLV were engulfed within the cytoplasm of AM, and there was a difference in the number of cytoplasmic fluorescent foci among AM (Figs. 2, 3). There were also many AM which had no cytoplasmic fluorescent foci (data not shown).

In Vivo Activation of AM by MDP-L18. As LUV are usually able to contain a larger amount of drug than MLV, LUV were used in the experiment. CHM-LUV accumulate in the lung more efficiently than conventional LUV after intravenous injection.

Mice were injected intravenously with different doses of MDP-L 18, either nonencapsulated or encapsulated within liposomes, ranging from 0.01 to 100 μg per mouse. CHM-LUV (MDP-L 18) led to a higher cytotoxic activity in AM than free MDP-L 18 at any dosage. A particularly significant increase in cytotoxicity was observed in the liposomes containing more than 1 μg of MDP-L 18, and maximal activation of AM was obtained in the liposomes containing 10 μg of MDP-L 18 (Fig. 4).

Next, AM were harvested four, six, 12, 24, 48 and 72 h after intravenous injection of

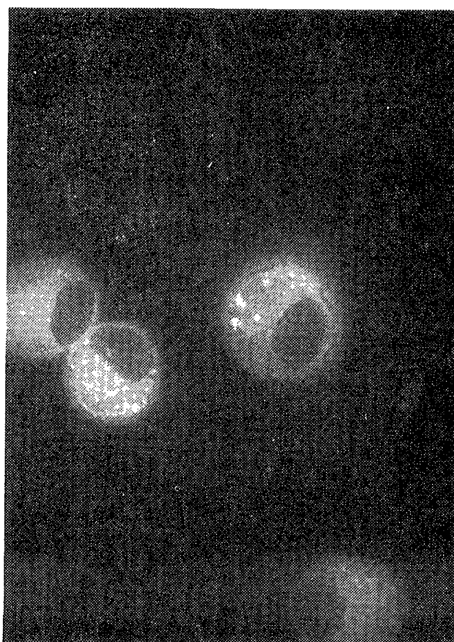

Fig. 2. Fluorescence micrograph of AM harvested one hour after intravenous injection of CHM-MLV labeled with terbium ; exposure time 1′ 00″. ×400.

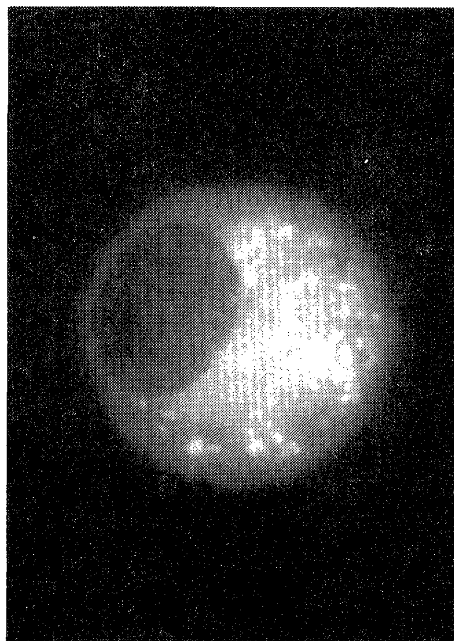

Fig. 3. Fluorescence micrograph of AM harvested 24h after intravenous injection of CHM-MLV labeled with terbium ; exposure time 3′ 20″. ×1,000.

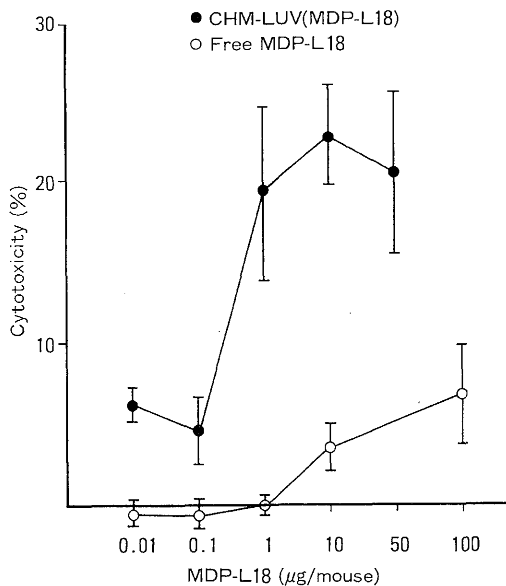

Fig. 4. The cytotoxic activity of mouse AM against 3LL by intravenous injection of MDP-L 18 ; Dose-response relationship. Groups of 4 mice were injected intravenously with different doses of MDP-L 18. AM were harvested 24h after the injection and were plated in culture wells. Next, ^{51}Cr-labeled 3LL were added. All experiments were done in triplicate cultures. Results are expressed as the mean ±SD of three experiments.

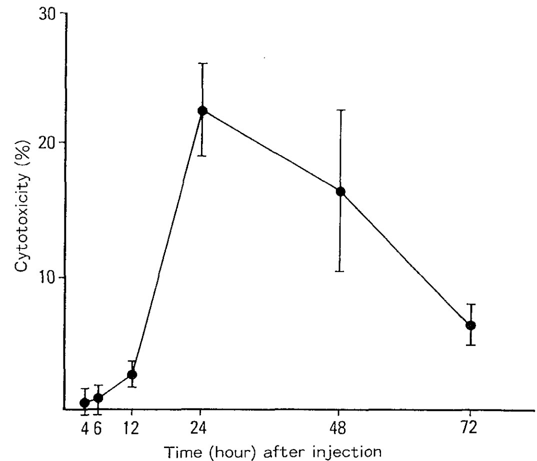

Fig. 5. Kinetics of the cytotoxic activity of mouse AM against 3LL by intravenous injection of CHV-LUV containing MDP-L18 (10 μg/mouse). AM were harvested at various times after the injection and were plated in culture wells. Next, ^{51}Cr-labeled 3LL were added. All experiments were done in triplicate cultures. Results are expressed as the mean ±SD of three experiments.

CHM-LUV (MDP-L 18 10 μg per mouse). The cytotoxic activity of AM reached a maximum 24 h after injection of the liposomes and decreased gradually with time, over the following 48 hour period (Fig. 5).

In Vivo Activation of AM by MA-CDA. MA-CDA itself had no cytolytic or cytostatic effect on 3LL in vitro (data not shown). Table 1 shows that the cytostatic activity of AM increased as the effector (AM) : target cell (3LL) ratio was increased. As shown in Table 2, the cytostatic activity of AM reached a maximum at three or five days after injection of MA-CDA, either nonencapsulated or encapsulated within liposomes. The activity of AM was highest in CHM-LUV (MA-CDA) among the groups studied. Even at seven days after injection, the activity in CHM-LUV (MA-CDA) was highest. These data demonstrate that MA-CDA encapsulated within CHM-LUV is more effective in the activation of AM than nonencapsulated MA-CDA.

Table 1. Cytostatic activity of mouse alveolar macrophages at different effector : target ratios

Treatment[a]	% cytostasis[b]		
E/T	5	10	20
PBS	6	8	18
CHV-LUV (MA-CDA 1000 μg)	29	41	73
Free MA-CDA 1000 μg	21	25	43

a) Groups of five mice were given intravenous injections of indicated materials. AM were harvested five days later.
b) All experiments were done in triplicate cultures. 10^4 target cells were added to AM monolayer.

Scanning Electron Microscopic Analysis of AM. *Control (untreated) AM.* After 24-h incubation on glass coverslips, most of the AM appeared more spherical than the activated AM, and were attached to coverslips by a small number of fine filopodia. A few AM were spread markedly over the coverslips. Furthermore, AM showed less surface activity

Table 2. Kinetics of the cytostatic activity of mouse alveolar macrophages against 3LL by intravenous injection of MA-CDA

Treatment[a]	% cytostasis[b]			
	Day 1	Day 3	Day 5	Day 7
PBS	4	16	16	15
CHM-LUV (MA-CDA 1000 μg)	9	86[c]	77[c]	34[c]
Free MA-CDA 1000 μg	9	47	53	0
empty CHM-LUV	10	13	12	8
PBS	0	7	13	14
CHM-LUV (MA-CDA 500 μg)	5	26[d]	36[d]	26[d]
Free MA-CDA 500 μg	4	12	20	8
empty CHM-LUV	ND*	10	9	ND

a) Groups of 5 mice were given intravenous injections of the indicated materials. AM were subsequently harvested at various times.
b) All experiments were done in triplicate cultures. Data represents % cytostasis at effector/target cell ratio of 20 : 1.
c) Statistically significant from Free MA-CDA 1000 μg, $p<0.05$.
d) Statistically significant from Free MA-CDA 500 μg, $p<0.01$.
* ND, not done.

than activated AM (Fig. 6). The control AM surface changed markedly with time in vitro : ruffles, lamellipodia, microvilli and filopodia disappeared after 48-h incubation (Fig. 7).

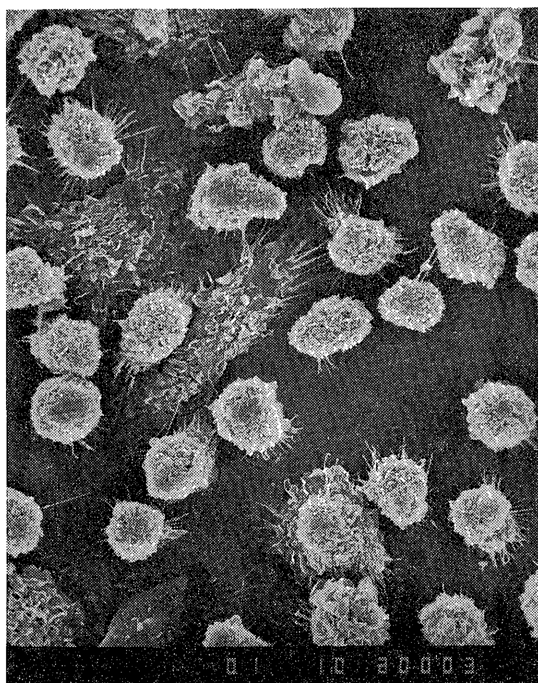

Fig. 6. Control (untreated) AM fixed after 24-h culture. ×1,000.

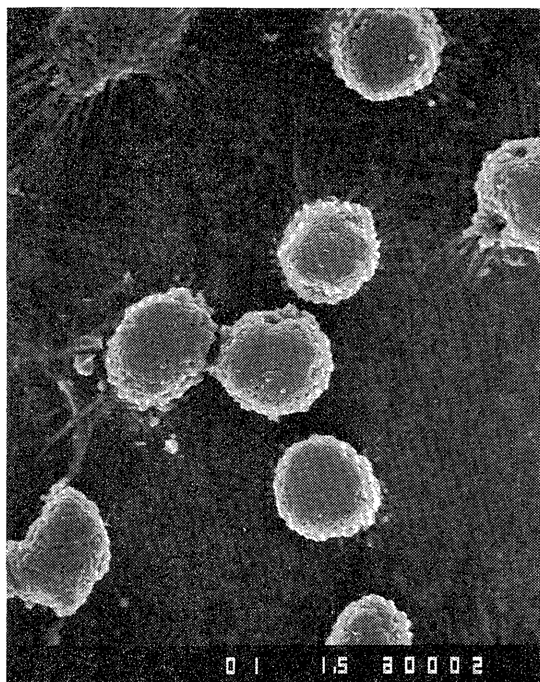

Fig. 7. Control AM fixed after 48-h culture. × 1,500.

AM exposed to MA-CDA in vitro. AM morphologically resembled activated AM in vivo (Fig. 8), and these morphological changes were maintained for 72 h. However, AM

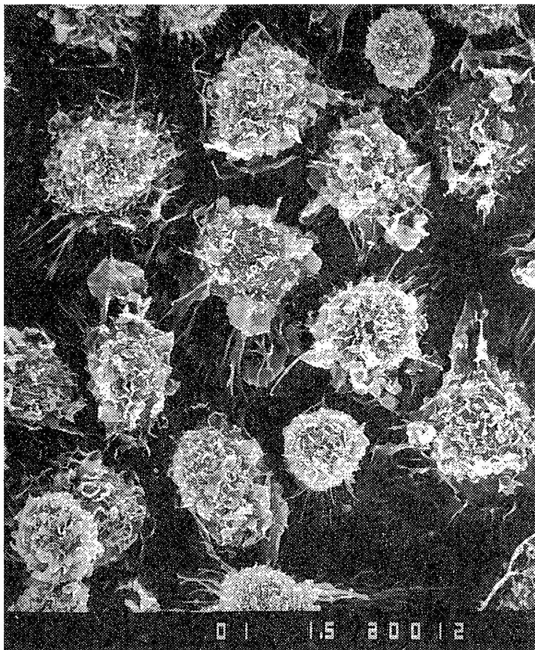

Fig. 8. AM cultured with MA-CDA 10 μg/ml in vitro for 72 h. ×1,500.

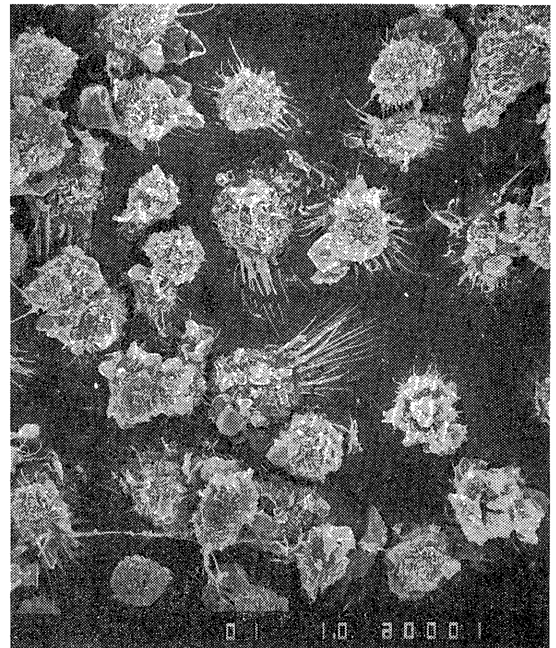

Fig. 9. Activated AM harvested three days after intravenous injection of uncapsulated MA-CDA 1,000 μg, 24-h culture. ×1,000.

activated by nonencapsulated MA-CDA in vitro had less cytostatic activity on 3LL than AM activated in vivo (data not shown)

Activated AM in vivo. AM harvested at three or five days after intravenous injection of nonencapsulated or encapsulated MA-CDA were more extensively spread over the coverslips and had a higher surface activity than control AM. Activated AM showed prominent ruffles, elongated filopodia, and thickened lamellipodia. It appeared that AM activated by encapsulated MA-CDA had higher surface activity than AM activated by nonencapsulated MA-CDA (Fig. 9-11).

Interaction between activated AM and target cells. After 24-h incubation of activated AM and target cells, the coverslips were vigorously washed in order to separate the two types of cells. The attachment between the two cell types was so strong that vigorous washing could not separate them (Fig. 12).

Treatment of Tumor-Bearing Mice by CHM-LUV (MA-CDA). Pulmonary metastases were first observed at 10 to 12 days after inoculation of 3LL, and existed microscopi-

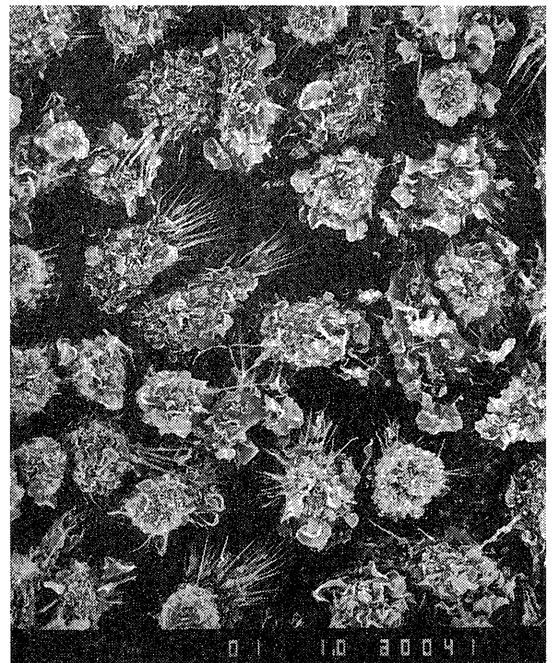

Fig. 10. Activated AM harvested three days after intravenous injection of CHV-LUV (MA-CDA 1,000 μg) ; 24-h culture. ×1,000.

Fig. 11. Activated AM harvested five days after intravenous injection of CHM-LUV (MA-CDA 1,000 μg), 24-h culture. ×1,000.

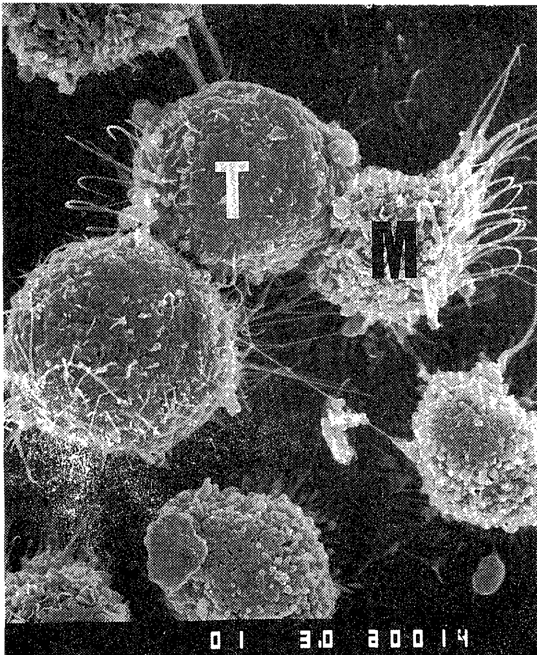

Fig. 12. Attachment between activated AM (M) and target cells (T). ×3,000.

cally at 14 days in most of the mice (data not shown). Results in Table 2 demonstrate that the cytostatic activity of AM in CHM-LUV (MA-CDA) was maintained for at least 5 days.

Next, inhibition of pulmonary metastases by CHM-LUV (MA-CDA) based on above results was attempted. A total dose of 3,000 μg MA-CDA per mouse was administered. The data of the survival experiment is shown in Fig. 13. Unfortunately, a difference in survival among the four treatment groups was not obtained, and all mice died of intrathoracic lymph node and multiple pulmonary metastases within day 30 of the experiment.

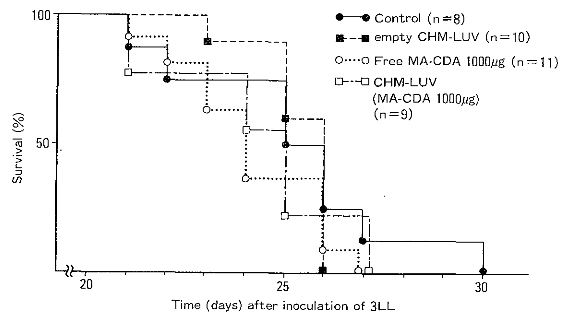

Fig. 13. Survival of 3LL-bearing mice treated with MA-CDA uncapsulated or encapsulated within CHM-LUV. Total dose of 3,000 μg MA-CDA per mouse was administred after inoculation of 3LL.

DISCUSSION

This study demonstrated that immunoactivator (MDP-L18 or MA-CDA) encapsulated within polysaccharide-coated liposomes enhanced the antitumor activity of mouse AM more effectively than the nonencapsulated immunoactivator (Fig. 4, Table 2). In addition, I reported, for the first time, that mouse AM had high surface activity upon treatment with synthetic polyanions such as MA-CDA, as visualized by scanning electron microscopy (Fig. 9-11). Macrophages activated by many types of immunoactivators for antitumor function have high antitumor activity, being specific for tumorigenic cells of any species (17-19). In the lung, AM plays an important role in defense against tumors (20). Therefore, it is advantageous to use AM activated by an

immunoactivator in the treatment of lung cancer. To date, there have been many studies using liposomes as drug carriers (2-5, 7-9), but few reports have employed immunoactivators encapsulated within liposomes in the treatment of cancer (6, 11).

For effective immunotherapy by AM targeting using liposomes, it is necessary for the liposomes to be engulfed by AM in vivo. In the present experiment, CHM-liposomes injected intravenously were obviously engulfed within AM (Fig. 2, 3, 4). However, LUV are unable to traverse the lung capillaries by themselves because of their large diameter (0.5-1.0 μm). It is considered that LUV in the lung capillaries were engulfed by circulating blood phagocytes which subsequently migrated to the alveoli to become AM, as Poste *et al.* reported (21). Therefore, it is considered that there were many AM which had no cytoplasmic fluorescent foci in pulmonary lavage fluid.

It is well known that MDP, or its derivatives, is capable of activating macrophages (22). Free MDP-L 18 injected intravenously did not lead AM to be cytotoxic at any doses ranging from 0.01 to 100 μg per mouse (Fig. 4). In contrast, MDP-L 18 encapsulated within CHM-LUV more than 1 μg enhanced the antitumor activity of AM. I speculate that the difference of the activation between free and encapsulated MDP-L 18 depends on whether MDP-L 18 sufficiently pass into AM following intravenous injectivn. For this reason, most of free MDP-L 18 injected intravenously may be excreted within a short time before MDP-L 18 pass into AM, and this short exposure of AM to MDP-L 18 may not be sufficient to activate AM. Parant *et al* reported that more than 50% of ^{14}C-MDP was recovered in the urine after 30 min, and more than 90% after 2 hours, following intravenous injection into mice (23). It was also proved, for the first time, that more than 24 hours are required to activate AM for antitumor function by encapsulated MDP-L 18, as shown in Fig. 5.

Polyanions have a broad range of biological activity including the ability to enhance host resistance against various microbial infections and cancer (24, 25). MA-CDA, which was used in this experiment, is also known to have the same ability by activating macrophages (12, 26). MA-CDA encapsulated within CHM-LUV enhanced the antitumor activity of mouse AM more effectively and for a longer period than nonencapsulated MA-CDA at the same dose (Table 2). The reason for this is that CHM-LUV efficiently carried MA-CDA to the alveolar space, so that MA-CDA encapsulated within CHM-LUV activated AM for antitumor function. CHM-LUV themselves had no ability to activate AM, as shown in Table 2. It was also considered that a concentration of MA-CDA sufficient to activate AM was maintained for several days by the encapsulation of MA-CDA within CHM-LUV.

It is interesting that a difference in time required to activate AM exists between encapsulated MDP-L 18 and MA-CDA. The difference may be due to a different process through which they activate AM, or a different mechanism involved in the destruction of tumor cells by activated AM. A resolution of its problem in the future can be expected.

Both in vivo and in vitro, mouse AM had very high surface activity due to MA-CDA such as prominent ruffles, elongated filopodia, and thickened lamellipodia. It is well known that the surface morphology of AM is changed by many kinds of agents (27, 28, 29) and cytokines (30), or some lung diseases (31). High surface activity may reflect activation of AM, but it does not always imply high antitumor activity. However, it was obvious in the case of MA-CDA that the antitumor activity of AM was related to high surface activity (Table 2, Figs 9-11). Futhermore, the attachment between activated AM and tumor cells was so strong that vigorous washing could not separate them (Fig. 12). It was clarified that macrophage-tumor binding might be one of mechanisms involved in the destruction of tumor cells by AM activated by MA-CDA.

I attempted inhibition of 3LL pulmonary metastasis by CHM-LUV (MA-CDA) based on the results shown in Table 2, but no prolonged survival was noted. Treatment of 3LL tumor-bearing mice with MA-CDA alone was not successful in various protocols. For this

reason, it was considered that MA-CDA did not activate AM sufficiently enough to enable AM to kill 3LL cells as was expected. In the past several years, attention has been focused on polyanions' ability to stimulate increased myelopoiesis in normal mice (32). Schlick *et al* reported the usefulness of treatment with polyanions and cyclphosphamide in tumor-bearing mice(33). It may be worthy to attempt future treatment with antineoplastic agents and CHM-LUV(MA- CDA).

In conclusion, I have reported that polysaccharide-coated liosomes containing immunotivators such as MDP-L18 or MA-CDA activated AM for antitumor function to a greater extent and for a longer time than free immunoactivator, and also that AM activated by MA-CDA encapsulated within these liposomes had high surface activity accompanied by antitumor activity.

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