# RATIONALE OF BIOLOGICAL RESPONSE MODIFIERS IN CANCER TREATMENT

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# Rationale of Biological Response Modifiers in Cancer Treatment

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# CONTENTS

# Preface

This monograph is a collection of contributions presented at the Sixth Symposium on the "Rationale of Biological Response Modifiers in Cancer Treatment", held at the Hakone Prince Hotel in Hakone, Japan, on August 31 and September 1, 1984. This symposium was designed to address the problem of the validity of in vitro and in vivo models for predicting clinical responses during the development and screening of new biological response modifiers (BRMs). Although extensive trials of immunotherapeutic treatment of cancer patients have been carried out in the last 15 years, results have not been satisfactory. Nevertheless, recent progress in the development of new synthetic immunomodulators and in gene technology and engineering have greatly stimulated basic and applied research on new and better BRMs.

In organizing this symposium, particular attention was also directed to the subject of the mechanisms by which BRMs modify the antitumor actions of the host in vivo. The exchange of new information and techniques related to the host's response to cancer and to approaches to the development of new therapeutic modalities was felt to be of major importance; all the participants were asked to address these points.

Scientific interest was great and discussion was lively throughout the two-day conference. I therefore believe that this conference achieved its scientific goals. I hope that this monograph will serve to promote the progress and development of research into BRM treatment of malignant disseminated diseases.

Finally, I wish to offer special thanks to the symposium coordinators, Dr. Saburo Sone, Mr. Yutaro Kaneko and Ms. Mitsuko Mori, who provided invaluable assistance during the meeting. We also owe a special debt of thanks to Ms. Hiroko Ishino, who extensively reviewed and edited all the manuscripts in order to enable rapid publication of the proceedings.

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# The use of liposomes as carriers of multiple biological response modifiers for systemic activation of macrophages

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## INTRODUCTION

The uncontrolled growth of metastases that are resistant to conventional therapies is a major cause of death from cancer.[1,2] Recent data from our laboratory and others indicate that metastases can arise from the nonrandom spread of specialized malignant cells that pre-exist within a primary neoplasm,[3] that metastases can be clonal in their origin,[4] that different metastases can originate from different progenitor cells,[4] and that, in general, metastatic cells can exhibit a higher rate of spontaneous mutation than benign nonmetastatic cells.[5] These data provide an explanation for the clinical observation that multiple metastases can exhibit different sensitivities to therapeutic modalities.[2] They imply that the successful therapy of disseminated metastases will have to circumvent the problems of neoplastic heterogeneity and the development of resistance to therapy by tumor cells.

Appropriately activated macrophages can fulfill these demanding criteria.[6] Macrophages can be activated to become tumoricidal by interaction with phospholipid vesicles (liposomes) containing various immunomodulators. Tumoricidal macrophages can recognize and destroy neoplastic cells *in vitro* or *in vivo*, while leaving non-neoplastic cells unharmed. Although the exact mechanism by which macrophages discriminate between tumorigenic and normal cells is unknown, it is independent of tumor cell characteristics, such as immunogenicity, metastatic potential, and sensitivity to cytotoxic drugs.[7] Moreover, macrophage destruction of tumor cells is apparently not associated with the development of tumor cell resistance.[6]

There are two major pathways to achieve macrophage activation *in vivo*. Frequently, macrophages are activated as a consequence of their interaction with microorganisms or their products, for example, endotox-

ins, the bacteria cell wall skeleton, and small components of the bacteria cell wall skeleton such as muramyl dipeptide (MDP).[8–10] *In vivo* activation of macrophages can also take place after their interaction with soluble mediators released by antigen- or mitogen-sensitized lymphocytes. The soluble lymphokine that induces macrophage activation is referred to as macrophage-activating factor (MAF). MAF first binds to a macrophage surface receptor and then is internalized to elicit tumoricidal properties in the macrophages.[11]

In general, attempts to specifically activate macrophages *in vivo* to enhance host defense against metastases have been unsuccessful. Systemic administration of MAF is hindered by the lack of purified preparations of this lymphokine and by the fact that lymphokines injected intravenously have a very short half-life and so do not activate macrophages *in vivo*.[12] The systemic activation of macrophages with microorganisms or their products has also suffered from major drawbacks. For instance, administration of whole bacteria such as Bacillus Calmette-Guerin activates various effector cells and is accompanied by serious toxicity problems such as allergic reaction and granuloma formation.[13] For this reason, little progress was made until the discovery of MDP, a small component of the bacterial cell wall that is capable of activating macrophages.[8,14] However, the use of water-soluble synthetic MDP is limited because, by 60 minutes after parenteral administration, this agent is cleared from the body to be excreted in the urine.[15] This brief period is insufficient to activate macrophages even under ideal *in vitro* conditions.[10]

Advances in liposome technology have provided a mechanism for activating macrophages *in situ* with soluble MAF or MDP or both. Liposomes can be used to carry agents to cells of the reticuloendothelial system, since these cells are responsible for the rapid clearance of particulate material from the circulation.[16–18] There are several advantages to using liposome-encapsulated materials to activate cells of the macrophage-histiocyte series *in vivo*. Many macrophage-activating agents such as bacterial products or lymphokines can be antigenic, and repeated systemic administrations can lead to adverse reactions. Liposomes consisting of natural phospholipids are nonimmunogenic, and thus the elicitation of allergic reactions commonly associated with the systemic administration of other immune adjuvants may be avoided.[18]

Numerous recent studies have shown that both MAF and MDP entrapped in liposomes are very efficient in activating macrophages to become tumoricidal *in vitro*.[6] Unlike free MAF, which requires binding to a macrophage surface receptor,[19] liposome-entrapped MAF enters the cytoplasm via phagocytosis and can activate macrophages that lack receptors for MAF.[20] Moreover, for activators such as MAF and MDP that are ordinarily degraded or cleared from the body too rapidly for

effectiveness, encapsulation in liposomes extends their active half-life within the body and enables these agents to activate macrophages *in situ*.[18,21–23]

Recent studies from our laboratory have shown that free or liposome-entrapped MAF and MDP can act synergistically to activate the tumoricidal properties in rat alveolar macrophages (AM) *in vitro*.[24] Since liposomes provide an efficient carrier vehicle for delivery of biologically active materials to macrophages *in vivo*,[25] activation of macrophages can be achieved to enhance host defenses against infections and cancer. In this report, we show that mouse AM can be rendered tumoricidal *in situ* by the intravenous injection of liposomes containing subthreshold doses of MAF and MDP, doses that are without effect when injected individually. Moreover, the repeated administration of these preparations is highly effective in eradicating large, established, spontaneous, pulmonary and lymph node melanoma metastases.

## MATERIALS AND METHODS

### Tumor culture

The B16BL/6 tumor line was obtained by an *in vitro* selection procedure for invasion.[26] The line originated from the B16 melanoma syngeneic to the C57BL/6 mouse. The cells were grown as monolayer cultures at 37°C in a humidified atmosphere containing 5% $CO_2$ in Eagle's minimum essential medium supplemented with 5% fetal bovine serum, vitamin solution, sodium pyruvate, L-glutamine, and nonessential amino acids (M.A. Bioproducts, Walkersville, Md). All cultures were free of mycoplasma and pathogenic mouse viruses.

### Animals

Eight- to ten-week-old specific pathogen-free male C57BL/6N mice were obtained from the NCI-Frederick Cancer Research Facility's Animal Production Area.

### Preparation of MAF

Cell-free supernatant fluids containing MAF activity were harvested from cultures of normal F344 rat lymphocytes incubated *in vitro* for 48 hours with Sepharose-bound concanavalin-A (Pharmacia, Piscataway, NJ), as

detailed previously.[18] The cell culture supernatant fluids were centrifuged and filtered through a 0.2 $\mu$m Millipore filter. The solution was either used immediately or stored at −20°C.

For convenience and brevity, such supernatant fluids are referred to as MAF throughout the remainder of this report.

### Reagents

The MDP was the kind gift of Ciba Geigy, Ltd. (Basel, Switzerland). All reagents used in our studies, such as media, MAF, MDP, and the final liposome preparations did not contain any endotoxins (detection limit of 0.125 ng/ml) as determined by the *Limulus* amebocyte lysate assay (Cape Cod Associates, Mass).

### Lipids and preparation of liposomes

Chromatographically pure distearoylphosphatidylcholine (PC) and phosphatidylserine (PS) were purchased from Avanti Biochemicals (Birmingham, Ala). Multilamellar vesicles (MLV) were prepared from a mixture of PC and PS (70/30 mol%). Various dilutions of MAF and MDP were encapsulated within the MLV, as described previously.[17] The liposome preparations were adjusted to a concentration of 12.5 $\mu$mol of total lipid/ml in $Ca^{++}$- and $Mg^{++}$-free Hanks' balanced salt solution (HBSS) and used within one hour.

### Determination of *in vivo* activation of AM

Normal mice were injected intravenously with 2.5 $\mu$mol of MLV that contained MAF or MDP, or both. In addition, control groups of mice were injected intravenously with HBSS, or with MLV that contained HBSS within their aqueous interior and that were suspended in HBSS containing free MAF or MDP, or both, at doses comparable to that entrapped within the MLV. Twenty-four hours after injection, the mice were killed, and their AM were harvested by tracheobronchial lavage.[10,24] Differential counts and nonspecific esterase staining revealed that over 95% of the lavaged cells were AM. The cells were plated into wells of Microtest II plates with a surface area of 38 mm² (Falcon Plastics Co., Oxnard, Calif). Nonadherent cells (< 10%) were removed by washing with medium 60 minutes after the initial plating.

### *In vitro* AM-mediated cytotoxicity

Macrophage-mediated cytotoxicity against B16 melanoma cells was assessed by a radioactive release assay, as described previously.[10,24] Ten thousand target cells were plated in each culture well to obtain an initial AM: target-cell ratio of 10:1. At this population density, normal (untreated) AM are not cytotoxic to neoplastic cells, whereas activated AM are. All cultures were re-fed 24 hours after the addition of tumor cells. The cultures were then incubated for two additional days. Three-day cultures were washed twice with HBSS, and the remaining adherent viable cells were removed from the wells with 0.1 ml of 0.5 N NaOH. The lysate was then monitored for radiation in a gamma counter. The percent cytotoxicity mediated by activated AM was calculated as follows:

$$\frac{\begin{array}{c}\text{cpm in target cells}\\ \text{cultured with}\\ \text{control AM}\end{array} - \begin{array}{c}\text{cpm in target cells}\\ \text{cultured with}\\ \text{activated AM}\end{array}}{\text{cpm in target cells cultured with control AM}} \times 100$$

The statistical significance of differences among the groups was determined by Student's two-tailed *t*-test.

### Treatment of spontaneous metastases by intravenous injection of liposomes containing MAF or MDP

To initiate spontaneous metastases, $2.5 \times 10^4$ viable B16BL/6 tumor cells were injected into one hind footpad of a mouse. When the local tumor had reached 10-12 mm in diameter (five to six weeks) the tumor-bearing leg, including the popliteal lymph node, was amputated at the midfemur. Seven days after the surgical removal of the primary tumor, mice with grossly visible spontaneous lung and lymph node metastases were given intravenous injections of liposomes (2.5 $\mu$mol of phospholipids suspended in 0.2 ml of HBSS). Each treatment group consisted of 18 to 20 mice. The liposomes contained various dilutions of either MAF or MDP, or both. Liposomes containing HBSS and suspended in HBSS containing MAF and MDP were used as control preparations. An additional control group included mice that received intravenous injections of HBSS alone. The mice were treated twice a week for four weeks and were monitored for up to 250 days. Dead or moribund animals were necropsied.

The Fisher exact test was employed to compare the proportion of survivors in one test group with that in another (control) group. The Cox test was used to compare the survival curve of one test group with that of

the controls or other groups.[27]

## RESULTS

### *In vivo* activation of AM by liposomes containing two immunomodulators

Mice were injected with control preparations or with MLV containing various agents. Twenty-four hours later, AM were harvested, and their ability to lyse syngeneic tumor cells was assayed *in vitro*. AM harvested from mice injected with HBSS, free MAF, free MDP or a combination of free MAF and MDP did not lyse the B16 melanoma cells (Table 1). In contrast, AM harvested from mice injected with liposomes containing MAF (diluted 1:2 or 1:10 in medium) were significantly cytotoxic against the B16 melanoma cells. When the MAF solution was diluted to 1:20 or 1:50, it did not generate tumoricidal activity in AM. Liposomes containing 6.25 or 0.6 $\mu$g of MDP led to a significant cytotoxic activity in AM, but liposomes with less MDP did not generate cytotoxic properties in the AM.

To determine the potential synergistic activation of AM by MAF and MDP encapsulated within the same liposomes, mice were injected intravenously with MLV containing various dilutions of MAF, various dilutions of MDP, combinations of MAF and MDP, or control preparations. AM were harvested 24 hours after treatment, and their tumoricidal properties were determined by the *in vitro* assay. MLV containing either a 1:20 dilution of MAF or a 1:20 dilution of MDP (equivalent to 0.3 $\mu$g of entrapped MDP) did not activate the AM. On the other hand, when MLV containing a 1:20 dilution of MAF and a 1:20 dilution of MDP (0.3 $\mu$g) were injected, significant *in situ* activation of AM occurred (Table 1).

These data indicate that the encapsulation of subthreshold doses of MAF (1:20 dilution) and MDP ($<0.3$ $\mu$g) within the same liposome leads to a significant ($p<0.001$) synergism in the *in situ* activation of AM.

The observed synergistic activation of liposome-entrapped MAF and MDP was dependent upon the delivery of both agents to the same macrophage. We base this conclusion upon the data shown in Table 1, which show that neither 2.5 $\mu$mol of MLV containing HBSS injected with a 1:20 dilution of unencapsulated MAF and a 1:20 dilution of unencapsulated MDP nor the injection of two separate liposome preparations (1.25 $\mu$mol of liposomes containing a 1:20 dilution of MAF admixed with 1.25 $\mu$mol of MLV containing a 1:20 dilution of MDP) activated AM

**Table 1** Synergistic activation of tumoricidal properties in murine alveolar macrophages by free MAF or MDP and by liposomes containing MAF or MDP or both

| Treatment of AM donors[a] | Percent AM-mediated cytolysis[b,c] |
|---|---|
| HBSS control | -7% |
| Free MAF (1:2) | 2% |
| Free MDP (6.25 $\mu$g) | 1% |
| Free MAF (1:2) and free MDP (6.25 $\mu$g) | -1% |
| MLV containing MAF (1:2) | 48%[d] |
| MLV containing MAF (1:10) | 26%[d] |
| MLV containing MAF (1:20) | -2% |
| MLV containing MAF (1:50) | -9% |
| MLV containing MDP (6.25 $\mu$g) | 45%[d] |
| MLV containing MDP (0.62 $\mu$g) | 41%[d] |
| MLV containing MDP (0.3 $\mu$g) | -1% |
| MLV containing MDP (0.12 $\mu$g) | -3% |
| MLV containing MAF (1:2) and MDP (6.25 $\mu$g) | 42%[d] |
| MLV containing MAF (1:20) and MDP (0.3 $\mu$g) | 44%[d] |
| MLV containing MAF (1:20) admixed with MLV containing MDP (0.3 $\mu$g) | 1% |
| MLV containing HBSS suspended in MAF (1:20) and MDP (0.3 $\mu$g) | 1% |

[a] At least 3 mice per group. Inoculum dose was 0.2 ml. The injection consisted of either free material or of 2.5 $\mu$mol of MLV. AM were harvested 24 hours later.

[b] $10^5$ AM were plated into 38 mm$^2$ culture wells. One hour later, the nonadherent cells were removed, and $10^4$ [$^{125}$I] IUdR-labeled B16 melanoma cells were added. The values are mean cpm $\pm$ SD in viable cells of triplicate cultures terminated after 72 hours of cocultivation.

[c] The percent cytolysis was calculated by comparison with control AM obtained from mice injected with HBSS.

[d] $p < 0.001$.

*in situ*.

**Eradication of spontaneous established lung and lymph node metastases by the intravenous injection of liposomes containing MAF or MDP or both**

The B16/BL6 melanoma line, which is syngeneic to C57BL/6 mice, was used to determine the effectiveness of liposome-encapsulated immunomodulators in the treatment of metastases. After implantation into the footpad, this tumor metastasizes to lymph nodes and the lungs in over 90% of recipient mice. C57BL/6 mice were given subcutaneous injections of $5 \times 10^4$ viable cells in 0.05 ml of HBSS. Four to five weeks later, the

tumor-bearing legs and the popliteal lymph nodes were amputated. Intravenous injections of various liposome preparations began seven days after the surgical removal of the primary melanoma. At this time, spontaneous pulmonary and lymph node metastases were well established, with some metastases reaching 1 mm in diameter. Liposomes were injected twice weekly for four weeks.

Practically all the mice receiving HBSS alone and MLV suspended in encapsulated MAF and MDP had died by Day 90 of the experiment (Fig. 1). On the other hand, multiple intravenous injections of liposomes containing either a 1:2 dilution of MAF or 6.25 $\mu$g of MDP resulted in long-term survival (>250 days) of 27% of the animals (5/18). Neither liposomes containing MAF at a 1:20 dilution nor liposomes containing MDP at doses lower than 0.6 $\mu$g were effective. In contrast, mice treated with liposomes containing both MAF and MDP at individual subthreshold concentrations resulted in enhanced survival rates, with 50% of the mice alive at day 250 ($p=0.0007$).

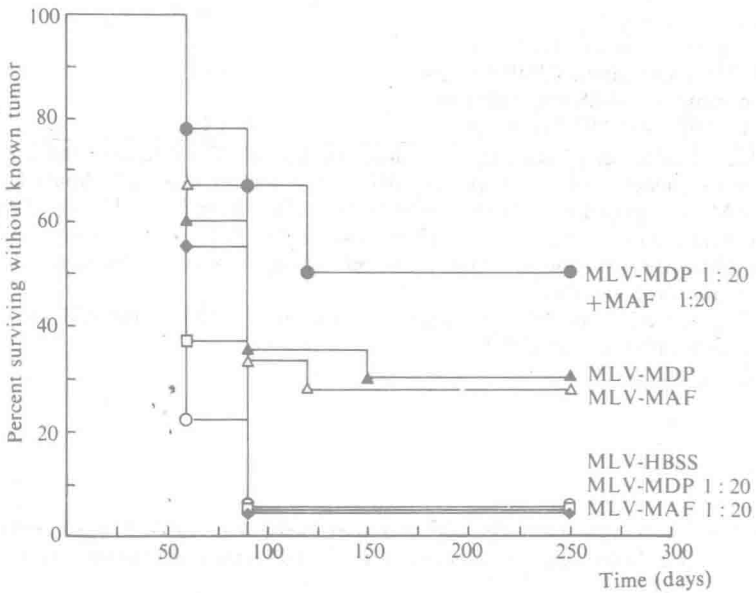


**Fig. 1** Treatment of spontaneous melanoma metastases by the systemic administration of liposomes containing immunomodulators. Mice (18-20 mice/group) received two intravenous liposome treatments per week for 4 weeks (8 injections). The difference in survival between the group treated with liposomes containing MAF and MDP and other treatment groups is highly significant ($p<0.0007$).
Time (days)
+MAF 1:20

## DISCUSSION

Advances in liposome technology have suggested a way in which immunomodulators might be used to activate macrophages *in vivo*. This involves encapsulating the agent in liposomes. There are several advantages to this approach: First, when injected intravenously, most liposomes localize in phagocytic cells of the reticuloendothelial system, cells responsible for the rapid clearance of particulate matter from the circulation. Second, encapsulation of macrophage activators within liposomes prevents their rapid degradation or clearance. Third, many macrophage-activating agents can be antigenic, and repeated systemic administrations can lead to undesirable side effects. Liposome encapsulation of agents can prevent such consequences.

Synergistic activation of the tumoricidal properties in AM by unencapsulated MAF and MDP has previously been shown to occur *in vitro*.[24] However, neither the intravenous injection of free MAF[18] nor of free MDP[21] led to systemic activation of macrophages *in situ*, whereas the intravenous administration of liposomes containing optimal doses of either MAF or MDP does generate AM tumoricidal properties *in vivo*[18, 21] (Table 1). On the other hand, intravenous administration of liposomes containing a 1:20 dilution of MAF or a 1:20 dilution of MDP did not activate mouse AM to become tumor cytotoxic, although when these two subthreshold doses of MAF and MDP were combined and encapsulated within the same MLV, significant *in situ* activation of AM occurred. Since neither diluted MAF nor diluted MDP entrapped in separate MLV activated AM *in situ*, the results obtained with their combination are evidence for synergistic rather than additive effects.

At the time systemic treatment of lung and lymph node metastasis with liposomes began, some spontaneous metastases were already visible. We deliberately postponed the start of treatment to Day 7 after surgery to allow examination of the hypotheses that MDP and MAF encapsulated within the same liposome would act synergistically to activate macrophages *in situ* and that this activation would increase the capacity of macrophages to destroy tumor cells, thus increasing long-term survival of the mice. Indeed, the results shown in Table 1 support these hypotheses. The intravenous injections of liposomes containing an optimal dose of MAF or MDP led to regression of metastases in at least 33% of the mice treated. Liposomes containing either diluted MAF or diluted MDP had no such effects. Furthermore, the intravenous injections of liposomes containing subthreshold amounts of both MAF and MDP were associated with a significant increase in long-term survival of mice (9 / 18,