Antitumor Effect of Vitamin D-Binding Protein-Derived Macrophage Activating Factor on Ehrlich Ascites Tumor-Bearing Mice (44339)

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Abstract. **Cancerous cells secrete α-N-acetylgalactosaminidase (NaGalase) into the blood stream, resulting in deglycosylation of serum vitamin D_3-binding protein (known as Gc protein), which is a precursor for macrophage activating factor (MAF). Incubation of Gc protein with immobilized β-galactosidase and sialidase generates the most potent macrophage activating factor (designated GcMAF). Administration of GcMAF to cancer-bearing hosts can bypass the inactivated MAF precursor and act directly on macrophages for efficient activation. Therapeutic effects of GcMAF on Ehrlich ascites tumor-bearing mice were assessed by survival time and serum NaGalase activity, because serum NaGalase activity was proportional to tumor burden. A single administration of GcMAF (100 pg/mouse) to eight mice on the same day after transplantation of the tumor (5×10^5 cells) showed a mean survival time of 21 ± 3 days for seven mice, with one mouse surviving more than 60 days, whereas tumor-bearing controls had a mean survival time of 13 ± 2 days. Six of the eight mice that received two GcMAF administrations, at Day 0 and Day 4 after transplantation, survived up to 31 ± 4 days whereas, the remaining two mice survived for more than 60 days. Further, six of the eight mice that received three GcMAF administrations with 4-day intervals showed an extended survival of at least 60 days, and serum NaGalase levels were as low as those of control mice throughout the survival period. The cure with subthreshold GcMAF-treatments (administered once or twice) of tumor-bearing mice appeared to be a consequence of sustained macrophage activation by inflammation resulting from the macrophage-mediated tumoricidal process. Therefore, a protracted macrophage activation induced by a few administrations of minute amounts of GcMAF eradicated the murine ascites tumor.** [P.S.E.B.M. 1999, Vol 220]

Inflammation results in macrophage activation leading to immune development. Inflamed tissues release membranous lipid metabolites, lysophosphatidylcholine (lyso-Pc), and other lysophospholipids that efficiently activate macrophages (1, 2). Inflammation of cancerous tissues (e.g., melanoma and bladder cancer) induced by intratumor administration of BCG (*Bacille Calmette Guerin*) or other bacterial cells can result in regression of local as well as metastasized tumors suggesting development of specific immunity against the tumor (3, 4). Inflamed cancerous tissues release alkylglycerols because cancerous cells contain alkylphospholipids (5, 6). Alkylglycerols are approximately 400 times more active macrophage stimulating agents than lysophospholipids in terms of the minimal dosages required for the optimal macrophage activation (6). This may explain why inflammation in cancerous tissues is curative. In fact, macrophages when activated have a potential to kill and eliminate cancerous cells (7–10).

The inflammation-primed macrophage activation process is the major macrophage activation cascade that requires participation of B and T lymphocytes (1, 2, 5, 6, 11) and serum vitamin D binding protein (DBP; human DBP is known as Gc protein) (12, 13). Stepwise hydrolysis of the

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This investigation was supported in part by US Public Health Service Grant AI-32140 and Elsa U. Pardee Foundation Grant to NY.

Received April 23, 1998. [P.S.E.B.M. 1999, Vol 220]
Accepted August 19, 1998.

0037-9727/99/2201-0020$10.50/0

trisaccharide of Gc protein with the inducible membranous β-galactosidase (Bgl_i) of inflammation-primed (or lyso-Pc-treated) B cells and the membranous *Neu*-1 sialidase of T cells generates a macrophage activating factor (MAF), a protein with N-acetylgalactosamine as the remaining sugar (13–15). Therefore, Gc protein is the precursor of MAF (13–16). Stepwise incubation of purified Gc protein with immobilized β-galactosidase and sialidase generates a remarkably high titered macrophage activating factor (16–20).

Advanced cancer patients are unable to activate their own macrophages because the precursor activity of serum Gc protein is lost (17, 18). This loss in the precursor activity results from deglycosylation of Gc protein by serum α-N-acetylgalactosaminidase (designated NaGalase) secreted from cancerous cells (17, 18). As the disease progresses, their serum enzyme levels increase with a concomitant decrease in the precursor activity of Gc protein (17, 18). GcMAF can bypass the deglycosylated (inactivated) Gc protein and directly act on macrophages for efficient activation (17, 19). GcMAF therapy of Ehrlich ascites tumor-bearing mice was monitored by serum NaGalase as a prognostic index along with ascites cell viability counts. Ehrlich ascites tumor-bearing mice receiving more than four administrations of GcMAF (100 pg/mouse) survived for at least 90 days (19). However, fewer than four administrations of GcMAF cured a certain fraction of the treated mice. Even with one or two administrations, one or two of eight mice survived. Macrophage-directed immunotherapy with these subthreshold administrations of GcMAF enabled us to investigate the curative mechanism of the treatment.

Materials and Methods

Animals and Tumor. Female BALB/c mice, 7–12 weeks of age, were obtained from the Jackson Labortories (Bar Harbor, ME). Mice were fed on Purina Mouse Chow and water *ad libitum.* Ehrlich ascites tumor was obtained from The Division of Cancer Treatment, National Cancer Institute (Bethesda, MD) and has been maintained by serial passages through BALB/c mouse peritoneal cavity. The Institutional Animal Care and Usage Committee approved the use of the animals for this study.

Proteins, Enzymes, and Reagents. Human Gc protein consists of three genetic groups (protein polymorphism): Gclf, Gcls and Gc2. Both Gclf and Gcls subtypes of Gcl carry sialic acid whereas Gc2 does not (13). Gcl protein (a mixture of Gclf and Gcls) was purified by vitamin D affinity chromatography (20, 21). Glycosidases were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and immobilized on Sepharose (20). p-Nitrophenyl N-acetyl-α-D-galactosaminide was purchased from Sigma Chemical Co. (St. Louis, MO). Freund's incomplete adjuvant was purchased from GIBCO BRL Life Technologies, Inc. (Grand Island, NY). Using the *Limulus* amebocyte lysate assay (1), we routinely tested for freedom of lipopolysaccharide contamination in the concentrated stock solutions of enzymes, human Gc protein, and culture media.

Cultivation of Ehrlich Ascites Tumor Cells. Ehrlich ascites tumor cells were cultured at 37°C in 35-mm wells containing medium RPMI-1640 supplemented with 10% FCS, 50 μg/ml penicillin and 50 μg/ml streptomycin in a 5% CO_2 incubator. Culture media were harvested and assayed for NaGalase activity.

Enzymatic Generation of Macrophage Activating Factor, GcMAF. Human Gc1 protein carries a trisaccharide composed of N-acetylgalactosamine with dibranched galactose and sialic acid termini (13–15). Stepwise digestion of purified Gcl glycoprotein (2 μg) by immobilized commercial β-galactosidase and sialidase (0.1 unit) yields the macrophage activating factor, GcMAF, a protein with N-acetylgalactosamine as the remaining sugar (20). A minute amount (10 pg/ml) of GcMAF was required to activate mouse peritoneal macrophages for 7-fold increased phagocytic and 15-fold increased superoxide generating capacities.

Treatment of Ehrlich Ascites Tumor-Bearing Mice with GcMAF. Since the Ehrlich ascites tumor was maintained by serial passage through the BALB/c mouse peritoneal cavity, the ascites tumor cells grew immediately when transplanted. To assess the fate of the transplanted tumor cells, GcMAF should be administered 6 hr post-transplantation but prior to the increase in cell counts. To avoid deglycosylation of GcMAF by highly concentrated α-N-acetylgalactosaminidase in the peritoneal cavity, GcMAF was administered subcutaneously in the lower dorsal flank. Approximately 3.5 hr post-administration of GcMAF, macrophages (including monocytes) were activated systemically and recruited to inflamed lesions.

Assay for α-N-acetylgalactosaminidase (NaGalase) in Sera of Ehrlich Ascites Tumor-Bearing Mice. Proteins in mouse sera (100 μl) were precipitated by 70% saturated ammonium sulfate. This high salt precipitation procedure also dissociates the product inhibitor from the enzyme. The precipitates were dissolved in 50 m*M* citrate phosphate buffer (pH 6.0) and dialyzed against the same buffer for 2 hr at 4°C. The dialysates were made up to 0.5 ml in volume and assayed for enzyme activity (17, 18, 22). The substrate solution (300 μl) contained 50 m*M* citrate buffer (pH 6.0) and 5 μmoles of p-nitrophenyl-N-acetyl-α-D-galactosaminide. Reaction was initiated by the addition of 500 μl of the dialyzed sample, incubated for 60 min at 37°C, and terminated by the addition of 200 μl of 10% TCA. After centrifugation, 500 μl of 0.5 *M* Na_2CO_3 solution was added to the supernatant. The amount of released p-nitrophenol was determined spectrophotometrically at 420 nm with Beckman DU640 spectrophotometer. Enzyme activity was expressed as nmoles/min/mg of serum protein.

The enzyme activity of control mouse sera was that of α-galactosidase, which hydrolysed the same chromogenic

substrate for NaGalase because these enzymes were evolutionarily conserved and shared 46% amino acid sequence homology (17, 18). The enzyme activities above the control mouse α-galactosidase activity (2.16 ± 0.15 nmole/min/mg) were considered to be NaGalase activity derived from cancerous cells (17).

Efficacy Assessment of GcMAF on Ehrlich Ascites Tumor-Bearing Mice. To evaluate the antitumor effect of GcMAF, BALB/c mice that received 5×10^5 tumor cells were singly or multiply injected with GcMAF ranging in doses from 0.5–5 ng/kg. Control mice received the vehicle only. To find the optimal GcMAF treatment schedule, the efficacy of GcMAF was expressed as survival time of animals by the percentage of the survival time of treated mice over that of untreated mice (23).

$$\%\ T/C = \frac{\text{mean survival time of treated mice}}{\text{mean survival time of untreated mice}} \times 100$$

Animals surviving more than 60 days were designated as long-term survivors or cured. One-half of the long-term survivors were sacrificed at 60–65 days for histological analysis (no tumor cell viability in the peritoneal cavity). The remainder was sacrificed at 90 or 150 days.

Efficacy of GcMAF was also assessed by time course analysis determining serum NaGalase activity following single or multiple administrations of GcMAF (5 ng/kg). Following administration of GcMAF, serum samples for NaGalase assays were collected at the indicated intervals. For each data point, three mice were anesthetized, and sera were collected from vena cava inferior. For ascites tumor cell counts in the peritoneal cavity, 8 ml of PBS were injected into the peritoneal cavity and aspirated with a syringe, and the total number of ascites tumor cells in peritoneal lavage was determined.

Treatment of GcMAF-Treated Tumor-Bearing Mice with Freund's Incomplete Adjuvant as an Inflammation-Inducer. During the tumoricidal process by activated macrophages, viability of the cancer cells decreased approximately 1000-fold in 3–4 days after a single GcMAF-treatment (19), and degeneration of the cancerous cells released lysophospholipids, which are inflammatory to the tissues. Lysophospholipids induce aseptic inflammation leading to macrophage activation (1). However, subthreshold concentrations of the inflammatory agent (e.g., lyso-Pc) cannot stimulate all of the animals to the same level. Freund's incomplete adjuvant is an inflammation-inducing agent (24). If the inflammatory-inducing agent were added to GcMAF-treated mice, all mice would be equally inflamed and stimulated for macrophage activation (24). Therefore, the tumor-bearing mice were treated once with 100 pg GcMAF, 3 or 5 days later, 1 μg of Freund's incomplete adjuvant was injected into the peritoneal cavity.

Results

Secretion of α-N-Acetylgalactosaminidase from Cultured Ehrlich Tumor Cells. Ehrlich ascites tumor cells grew exponentially. The doubling time of the tumor, computed by cell counts, was approximately 22 hr. The profile of the time course of NaGalase enzyme levels in culture media of 5×10^5 and 1×10^6 Ehrlich tumor cells/well is shown in Figure 1. The enzyme activity increased linearly with time. Thus, cultured Ehrlich ascites tumor cells secreted NaGalase into the culture medium as tumor cells grew.

Correlation of Serum α-N-Acetylgalactosaminidase (NaGalase) Activity with Tumor Burden in Mice Transplanted with Ehrlich Ascites Tumor Cells. When mice were transplanted with 5×10^5 Ehrlich ascites tumor cells, serum NaGalase activity increased as the ascites tumor cells grew in the peritoneal cavity. As shown in Figure 2, serum NaGalase activity in mice bearing Ehrlich ascites tumor increased as the tumor cell counts in the peritoneal cavity increased logarithmically. There is a statistical deviation with these two parameter measurements ($r = 0.7496$, $P < 0.05$). Because of linearity of the arithmetic serum NaGalase activity increment versus the logarithmic cell count increment, serum NaGalase increased as the tumor cell concentrations in the peritoneal cavity increased logarithmically. This observation implies that, at the higher tumor cell concentrations in the peritoneal cavity, the metabolic capacity of the tumor cells to produce NaGalase decreased as the tumor cell counts increased due to a cell crowding effect.

Antitumor Effect of the Enzymatically Generated Macrophage Activating Factor, GcMAF, on Mice Transplanted with Ehrlich Ascites Tumor. When eight BALB/c mice were transplanted with 5×10^5 Ehrlich ascites tumor cells and 6 hr later were administered with GcMAF (5 ng/kg), seven mice survived for 21 ± 3 days whereas one mouse survived for more than 60 days (Table I). On the other hand, all of the control mice that received the ascites tumor died within 13 ± 2 days. However, when these mice received an additional administration

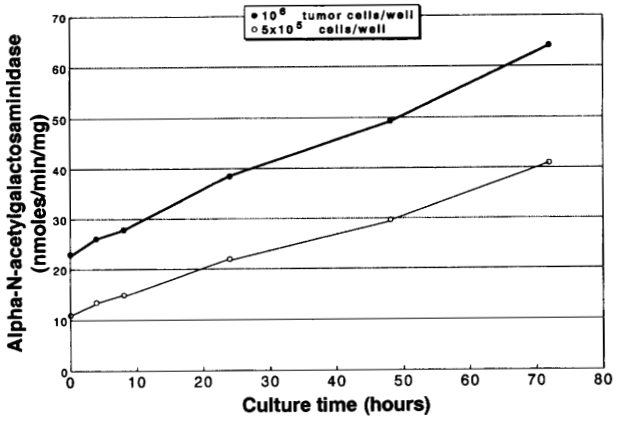

Figure 1. Time course analysis of secretion of α-N-acetylgalactosaminidase (NaGalase) into culture medium from cultured Ehrlich ascites tumor. Two different cell numbers, 5×10^5 and 10^6 cells/well were used. The data are expressed as mean values in three independent experiments.

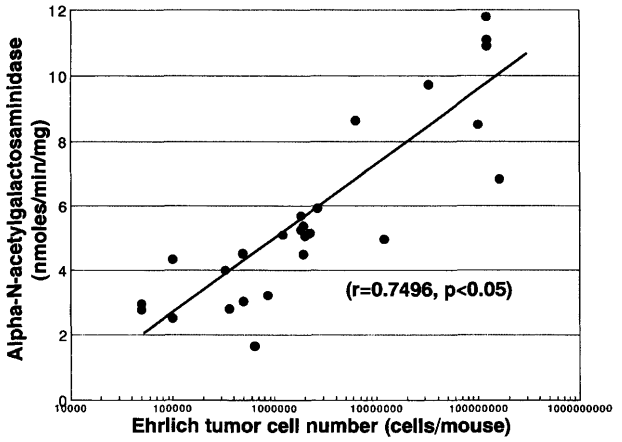

Figure 2. Correlation between serum NaGalase and Ehrlich tumor cell counts in the peritoneal cavity.

Table I. Therapy of Mice Bearing Ehrlich Ascites Tumor with the Enzymatically Generated Macrophage Activating Factor, GcMAF

Number of mice treated	Post-transplant treatment time (days)	Number of mice/survived	Survival period[a] (days)
8	Untreated control	8	13 ± 2
8	Day 0	7	21 ± 3
		1	>60
8	Day 0 and 4	6	31 ± 4
		2	>60
8	Day 0, 4 and 8	2	38 ± 8
		6	>60

Note. 5×10^5 tumor cells/mouse: 100 pg GcMAF/mouse.
[a] Survival period, values represent mean ± SEM of triplicate data. The results were repeated three times using either BALB/c or Swiss mice.

of GcMAF at 4 days post-transplantation, six of eight mice survived for 31 ± 4 days while two mice survived for more than 60 days. Of the mice that received three GcMAF administrations, at Days 0, 4, and 8 post-tranplantation, two survived for 48 ± 6 days, and the remaining six survived for at least 60 days.

Conditions for GcMAF Therapy of Ehrlich Ascites Tumor. Antitumor effects of GcMAF on mice bearing Ehrlich ascites tumor can be expressed as % T/C values (23) as shown in Table II. We first compared the efficacy of single GcMAF administration under different conditions (i.e., dosage, time, and route of administrations). The optimal antitumor effect of single GcMAF administration was achieved by subcutaneous administration of GcMAF (5 ng/kg) at 6 hr post-transplantation.

Figure 3 illustrates the % T/C values of single and multiple GcMAF administrations. The % T/C values of one and two GcMAF treatments were 161 and 238, respectively. The % T/C value of three GcMAF treatments for two mice, excluding six mice for 60 day survivors, was 309. Thus, three GcMAF administrations were most effective for treatment of Ehrlich ascites tumor.

Table II. Antitumor Effects of Single GcMAF Administration on Mice Bearing Ehrlich Ascites Tumor Under Various Conditions

Various GcMAF treatment condition	Survival time index (% T/C index[a])	Number of mice cured (8 mice/group)[b]
Untreated	100[c]	
GcMAF dosage (intramuscular)		
0.5 ng/kg	123	0
2.5 ng/kg	145	1
5.0 ng/kg	157	1
Timing for administration (5 ng/kg, intramuscular)		
Day 0	169	1
Day 1	122	0
Route for administration (5 ng/kg)		
subcutaneous	157	1
intraperitoneal	118	0

Note. These results were repeated three times using either BALB/c or Swiss mice.
[a] % T/C = (mean survival of treated mice/mean survival of untreated mice) × 100. This computation excluded long-term (>60 days) survivors.
[b] Long-term survivors more than 60 days.
[c] Mean survival of untreated Ehrlich ascites tumor bearing mice was 13.1 ± 0.7 days.

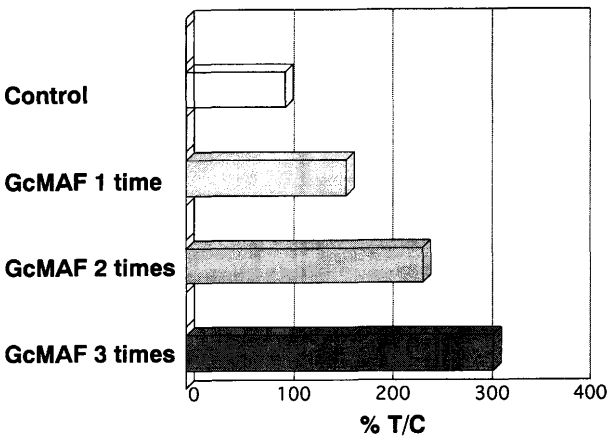

Figure 3. The % T/C values of multiple GcMAF treatments of mice bearing Ehrlich ascites tumor. Mice were treated with GcMAF (5 ng/kg) subcutaneously starting 6 hr after transplantation of 5×10^5 Ehrlich ascites tumor cells/mouse and thereafter at 4-day intervals (Day 4 and Day 8). The long-term survivors were excluded from % T/C computation.

Time course analyses with the percentage survival of mice with multiple GcMAF treatments are shown in Figure 4. Two or three of eight mice survived over 60 days after one or two administrations of GcMAF (5 ng/kg), respectively, whereas all of the untreated mice died within 14 days. These results were highly reproducible (see Tables I and II). With mice that received three GcMAF administrations with 4-day intervals, six of eight mice showed an extended survival period of over 60 days. Thus more than three GcMAF administrations were required for complete eradication of the ascites tumor (19) although only one or

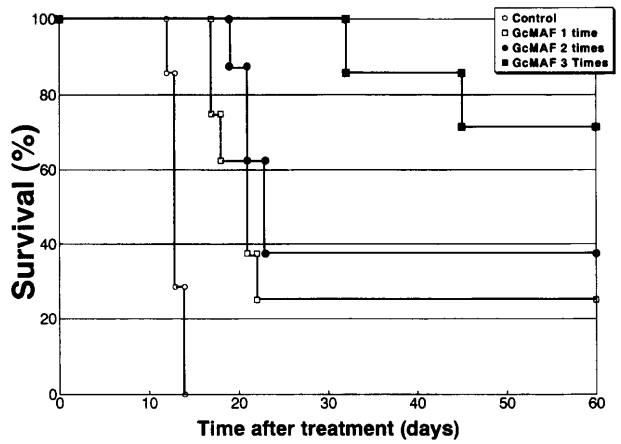

Figure 4. Time course study of the percentage of survivors of single or multiple GcMAF (5 ng/kg) treatments of mice bearing Ehrlich ascites tumor.

two administrations of GcMAF enabled the survival of a few mice for more than 60 days.

Therapeutic Effect of GcMAF On Mice Bearing Ascites Tumor Cells was Monitored by Serum α-N-Acetylgalactosaminidase Activity. The time course profile of serum NaGalase activity of tumor-bearing mice that were treated with one, two, and three GcMAF administrations is shown in Figure 5. For each treatment schedule, serum enzyme activity assay started 2 days after the last injection. Four to six days after the last injection for each treatment schedule, a brief rise followed by a decrease in serum NaGalase activity was observed. This sudden drop in enzyme activity appeared to be the result of cytocidal action on the cancerous cells because the activated macrophage cell counts increased 30- to 40-fold at 4 days post-administration of GcMAF (20, 25). With three GcMAF administrations 16–60 days after treatment, a very low level of serum NaGalase (2.61 ± 0.11 nmole/min/mg) was detected in the majority (six out of eight) of the treated mice. Therefore, serum NaGalase levels of the three-GcMAF-treatments group were as low as those of healthy control mice throughout the survival period.

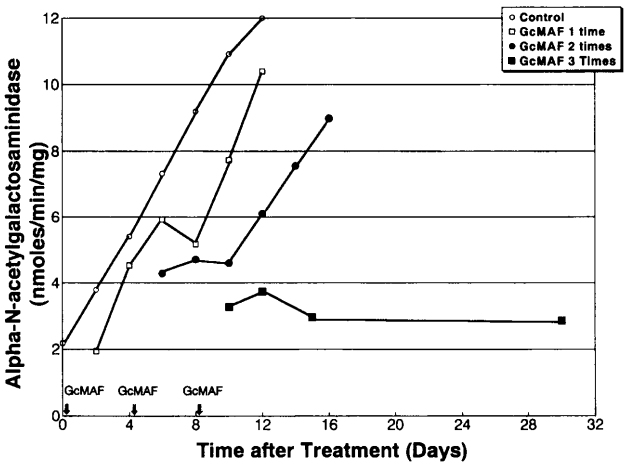

Figure 5. Time course analysis of serum NaGalase assays after single or multiple GcMAF (5 ng/kg) treatments on Ehrlich ascites tumor-bearing mice. The data are expressed as mean values of triplicate assays.

Effect of Inflammation on GcMAF-Treated Mice Bearing Ascites Tumor Cells. Tumoricidal and phagocytic processes by the activated macrophages release lysophospholipids that induce an inflammatory reaction in the lesions (1, 2). However, the extent of inflammatory response to subthreshold levels of lysophospholipids varies among individual animals. This can be demonstrated by macrophage activation at Day 7 or later after a single GcMAF treatment since this time point GcMAF-driven macrophage activation ceases, and only lysophospholipid-induced macrophage activation can be measured (Yamamoto N *et al.*, unpublished data). Since Freund's incomplete adjuvant is an inflammation-inducing agent (24), it was injected into the peritoneal cavity of mice 3 or 5 days after GcMAF treatment. Although single GcMAF treatment of the tumor-bearing mice cured one or two of the eight mice (Table I and Figure 4), injection of Freund's incomplete adjuvant into the peritoneal cavity cured the disease from all of the mice that received a single GcMAF treatment (as shown in Table III). Therefore, a sustained inflammatory response to Freund's incomplete adjuvant appears to eradicate tumor cells in mice that are treated with even a single GcMAF dose.

Discussion

Since GcMAF can bypass the deglycosylated Gc protein, macrophages of cancer-bearing hosts are capable of being activated 3.5 hr after GcMAF administration as demonstrated by greatly enhanced phagocytic and superoxide generating capacities (19). These activated macrophages are tumoricidal as demonstrated by *in vitro* cytocidal assay for Ehrlich ascites tumor (Yamamoto *et al.*, unpublished). GcMAF is also highly mitogenic and stimulates the progenitor cells thereby resulting in a dramatic increase in cell counts of the activated macrophages up to 30- to 40-fold in 4 days following GcMAF administration (24, 25). These activated macrophages are recruited at the inflamed lesions. Therefore, the most beneficial therapeutic regimen consists of treatment that should be given at 4-day intervals. If the ascites tumor growth was repeatedly suppressed by three administrations of GcMAF, the ascites tumor in the majority of mice (e.g., six of eight mice) did not grow back for at least 60 days. Protracted suppression of tumor growth by four GcMAF administrations appeared to be required for immune development against the ascites tumor (19). However, even one or two administrations of GcMAF cured one or two of the eight mice, respectively. Because the half-life of the activated macrophages is approximately $5\frac{1}{2}$ days (11), one administration of GcMAF activates macrophages for 5 days. Beyond this period, macrophage-directed cell killing of cancerous cells can lead to inflammation. During tumoricidal and phagocytic processes, the degenerative process of cancerous cells releases lysophospholipids. These lysophos-

Table III. Effect of Inflammation on GcMAF-Treated Mice Bearing Ehrlich Ascites Tumor is Assessed by Peritoneal Tumor Cell Viability and Mouse Survivals

Tumor + GcMAF	Administration of adjuvant (days post-GcMAF)	Viable tumor cells (days assayed)	Mouse survived (days)
Tumor only		1×10^8 (14)	16 ± 4
Tumor + GcMAF	None	3.22×10^5 (14) 3.52×10^7 (20)	23 ± 5
Tumor + GcMAF	Freund's adjuvant (3 day post-GcMAF)	$<5.0 \times 10^3$ (20)	>60
Tumor + GcMAF	Freund's adjuvant (5 day post-GcMAF)	$<5.0 \times 10^3$ (20)	>60
Tumor + No MAF	Freund's adjuvant[a]	4.58×10^7 (14)	18 ± 3

Note. BALB/c mice were intraperitoneally transplanted with 5×10^5 Ehrlich ascites tumor and 6 hr later treated with 100 pg GcMAF/mouse subcutaneously at lower dorsal. At 3 or 5 days after tumor transplantation/GcMAF treatment, Freund's incomplete adjuvant was administered into the peritoneal cavity. The viable tumor cell counts in the peritoneal cavity were determined at the indicated time. The number of viable tumor cells/ml in the peritoneal cavity is the mean value of triplicate assays. Mosue survival in days is expressed as the mean value of quadruplicate ± SEM.

[a] Time of adjuvant administration is 3 days post-transplantation of the tumor.

pholipids in turn can stimulate lymphocytes to generate macrophage activating factor if the glycosylated Gc protein is available (12, 20). However, subthreshold concentration of lysophospholipids can stimulate a fraction of these animals. Because tumor cell counts decrease 1000-fold after each GcMAF treatment while the NaGalase activity decreases greatly, lyso-Pc-primed lymphocytes convert the glycoslylated peritoneal Gc protein to the macrophage activating factor. Thus, some mice continue to develop the tumoricidal process by macrophages activated as a result of inflammation (i.e., production of lysophospholipids). This may explain why one or two mice among eight mice that received one or two GcMAF treatments survived for more than 60 days. If Freund's incomplete adjuvant as an inflammation-inducer (24) was injected into the peritoneal cavity of mice that received one GcMAF treatment, macrophages in all the mice were activated for a prolonged period leading to curing of the disease. However, if Freund's incomplete adjuvant was injected into the peritoneal cavity of the mice that had not received GcMAF treatment, the macrophages could not be activated because of the presence of a larger amount of NaGalase and the absence of glycosylated Gc protein. Therefore, treatment of the ascites tumor-bearing mice with Freund's incomplete adjuvant alone was not curative (see Table III).

GcMAF, as compared to the long list of cytokines is the most potent cancer therapeutic remedy we have ever encountered (19, 26, 27). Significance of the GcMAF therapy has been greatly broadened and enhanced by the discovery of the prognostic serum NaGalase. Because serum NaGalase activity is proportional to tumor burden in the hosts (18, 19), serum NaGalase activity appears to be a precise prognostic index for not only macrophage-directed immunotherapy but also chemotherapy, radiation (17, 28), and photodynamic therapy (28).

The authors dedicate this study to the memory of Dr. Stephen R. Max, Director for Research and Development, Albert Einstein Medical Center, Philadelphia, PA 19141.

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