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Structurally well-defined macrophage activating factor derived from vitamin D_3-binding protein has a potent adjuvant activity for immunization

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Summary Freund's adjuvant produced severe inflammation that augments development of antibodies. Thus, mixed administration of antigens with adjuvant was not required as long as inflammation was induced in the hosts. Since macrophage activation for phagocytosis and antigen processing is the first step of antibody development, inflammation-primed macrophage activation plays a major role in immune development. Therefore, macrophage activating factor should act as an adjuvant for immunization. The inflammation-primed macrophage activation process is the major macrophage activating cascade that requires participation of serum vitamin D_3-binding protein (DBP; human DBP is known as Gc protein) and glycosidases of B and T lymphocytes. Stepwise incubation of Gc protein with immobilized β-galactosidase and sialidase efficiently generated the most potent macrophage activating factor (designated GcMAF) we have ever encountered. Administration of GcMAF (20 or 100 pg/mouse) resulted in stimulation of the progenitor cells for extensive mitogenesis and activation of macrophages. Administration of GcMAF (100 pg/mouse) along with immunization of mice with sheep red blood cells (SRBC) produced a large number of anti-SRBC antibody secreting splenic cells in 2–4 days. Thus, GcMAF has a potent adjuvant activity for immunization. Although malignant tumours are poorly immunogenic, 4 days after GcMAF-primed immunization of mice with heat-killed Ehrlich ascites tumour cells, the ascites tumour was no longer transplantable in these mice.

Key words: adjuvant, antibody secretion, inflammation, macrophage activating factor, macrophage activation, mitogenesis, progenitor, vitamin D_3-binding protein.

Introduction

Adjuvants are substances that non-specifically enhance the immune response to antigen. The most frequently used adjuvants are water-in-oil emulsions with the antigen in the aqueous phase (e.g. Freund's incomplete adjuvant). The adjuvant properties are further enhanced by the addition of a microbial antigen to the mixture (e.g. heat-killed tubercle bacilli in Freund's complete adjuvant).[1,2] The action mechanism of adjuvants is poorly understood. The antigen in emulsion is resistant to dispersal, and it therefore acts as a depot for prolonged antigen stimulation. Thus, at least part of the immunopotentiating capacity of adjuvants is thought to be due to the slower release of antigen.[1,2] This is not the major mechanism of action, however, because Freund's incomplete adjuvant (minus bacteria) is a poor substitute for the complete form.[1] Adjuvants may relate to their ability to cause inflammation, thereby intensifying the general reaction (chemotaxis and activation of antigen-presenting cells) to antigen exposure.[1,3,4] Particularly, heat-killed tubercle bacilli in the adjuvant (i.e. Freund's complete adjuvant) presumably induce severe inflammation that appears to play a key role in potentiation of antibody production. Thus, adjuvants do not have to be mixed with antigens and the site of adjuvant administration may not have to be the same site of antigen injection for enhancement of antibody production as long as inflammation is induced in the hosts. However, antigen response is more effective if inflammation is induced at the site of antigen injection because chemotaxis enhances antigen processing.

Microbial infections produce inflammation. Inflamed lesions attract and activate macrophages and induce immunity against the infectious agents, implying that biochemical signals should be radiating out from the inflamed lesions. Inflamed tissues produce lysophospholipids.[5–8] These lysophospholipids are potent chemotactic and macrophage stimulating agents.[7–9] Thus, administration of lysophospholipids (5–20 μg/mouse) produces aseptic inflammation and activates macrophages to phagocytize target antigens or cells via the Fc receptor[5–11] and to generate superoxide.[12,13] *In vitro* treatment of mouse peritoneal adherent cells (macrophages) with lysophosphatidylcholine (lyso-Pc) results in no enhancement of macrophage phagocytic activity[5–10] and superoxide generating capacity.[12,13] However, incubation of peritoneal cells (mixture of adherent and non-adherent cells) with lyso-Pc in a serum-

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supplemented medium for 30 min, followed by 3 h cultivation of adherent cells alone, markedly enhances Fc receptor-mediated phagocytic and superoxide generating capacities of macrophages,[9–13] implying a participation of non-adherent (B and T) cells and a serum component in the activation of macrophages.[6,9–15]

Analysis of macrophage activating signal generation from the non-adherent cells showed that vitamin D_3-binding protein (DBP; human DBP is known as group-specific component, Gc) is a serum factor required for the inflammation-primed activation of macrophages.[16–20] Studies of intercellular signal transmission among non-adherent (B and T) cells revealed that Gc protein is modified by membranous β-galactosidase of inflammation-primed (or lyso-Pc-treated) B cells and membranous sialidase of T cells to yield the macrophage activating factor[14,20,21] (Fig. 1a). Incubation of Gc protein with a mixture of commercial β-galactosidase and sialidase efficiently generated the macrophage activating factor (designated GcMAF).[17,20] However, for the *in vivo* application of GcMAF, contamination of the enzymes in the GcMAF inoculum can be avoided if immobilized enzymes are used for preparation of GcMAF. Stepwise incubation of Gc protein with immobilized β-galactosidase and sialidase efficiently generated probably the most potent macrophage activating factor, GcMAF (Fig. 1b) ever discovered.[21] A 3 h incubation of mouse peritoneal macrophages with picomolar amounts of GcMAF resulted in a greatly enhanced phagocytic activity.[14] Administration of a minute amount (4–50 pg/mouse) of the enzymatically generated macrophage activating factor, GcMAF, to mice resulted in greatly enhanced (five to seven-fold) phagocytic and (15–30-fold) superoxide generating capacities of peritoneal macrophages.[14,20,22]

In the present communication, we discuss that administration of adjuvants generates macrophage activating factor that acts on the progenitor cells for activation and mitogenesis of macrophages. We report that the structurally defined macrophage activating factor, GcMAF, has a potent adjuvant effect on antibody production.

Materials and methods

Mice

Female BALB/c mice, 7–12 weeks of age weighing ~20 g, were obtained from Jackson Laboratories, Bar Harbor, ME, USA. Mice were fed Purina Mouse Chow and water *ad libitum*.

Chemicals and reagents

Freund's incomplete adjuvant and Freund's complete adjuvant were purchased from Gibco BRL Life Technologies, Inc. Grand Island, NY, USA. Phosphate-buffered saline containing 1 mmol/L or 0.1 mmol/L sodium phosphate and 0.15 mol/L NaCl, was prepared without addition of Ca^{2+} and Mg^{2+} ions. Human Gc protein consists of three genetic groups (protein polymorphism): Gc1f, Gc1s and Gc2. Both Gc1f and Gc1s subtypes of Gc1 carry sialic acid while Gc2 does not.[23,24] Gc1 protein was used for the present study because of the major isoform of Gc protein. Gc1 (a mixture of Gc1f and Gc1s) was purified by vitamin D-affinity chromatography.[25] Glycosidases were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN, USA. To avoid contamination with enzymes in GcMAF preparation, immobilized glycosidases were prepared by the use of cyanogenbromide-activated Sepharose 4B gel (Pharmacia Biotech Inc., Piscataway, NJ, USA).[21] Stepwise treatment of Gc protein with the immobilized β-galactosidase and sialidase generated the most potent macrophage activating factor, GcMAF.[21]

Culture media

For manipulation *in vitro* and cultivation of mouse peritoneal cells and adherent and non-adherent cells, medium RPMI-1640 supplemented with 0.1% egg albumin (EA medium) was used. Eagle's medium was used for Jerne plaque assay.[26]

Treatment of mice with Freund's complete or incomplete adjuvant

Freund's complete or incomplete adjuvant (1 μg/mouse) was injected at subcutaneously lower dorsal or into the peritoneal cavity

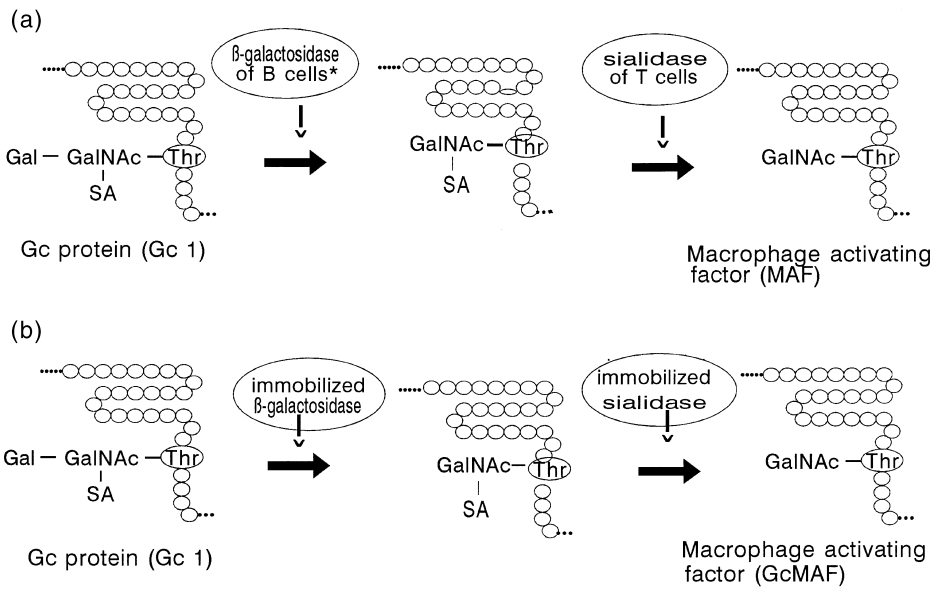

Figure 1 Schematic illustration of the formation of macrophage activating factor from vitamin D_3-binding protein (Gc protein) by (a) the action of lymphocyte glycosidases and (b) stepwise treatment of Gc protein with immobilized glycosidases to generate the macrophage activating factor (GcMAF).

of BALB/c mice. At various intervals (e.g. day 2 and 4 or 5), peritoneal macrophages were harvested as described previously.[9–11] Briefly, 8 mL of 0.1 mmol/L PBS containing 0.15 mol/L NaCl were injected into the peritoneal cavity. After 10 s abdominal massage, peritoneal lavage was aspirated, washed and assayed for cell counts and the superoxide generating capacity of macrophages.[12,13]

Treatment of mice with GcMAF

The mice had a total of 100 pg GcMAF once or 20 pg GcMAF daily administered to the peritoneal cavity or subcutaneously lower dorsal. Age-matched sham control mice were injected with equal volumes (0.3 mL) of sterile pyrogen-free saline. Two and 4 days after the first administration, peritoneal macrophages were harvested and assayed for cell counts and superoxide generating capacity.[12,13]

Assay for antibody secreting cells

A modified procedure of Jerne *et al*. was used to quantify the number of IgM antibody secreting cells (plaque-forming cells (PFC)) in the spleens of GcMAF-treated mice.[26] Mice were treated with various amounts of GcMAF and immunized with intraperitoneal injection of sheep erythrocytes (8×10^8 cells/mouse) 6 h after GcMAF administration. Spleen cells were harvested on days 1, 2, 3, 4 and 5 post-administration of sheep erythrocytes (SRBC). After washing, cell number was adjusted to $\sim4\times10^7$ cells/mL. Various aliquots (5, 10, 25, 50 and 100 μL) were admixed with 500 μL of molten agar (0.7% agarose in Eagle's medium at 45°C) and 50 μL containing 15% SRBC in small tubes at 45°C. The mixture was immediately poured onto agarose pre-coated slides (75×20 mm) and spread evenly. After several minutes, the slides were placed on wet filter papers in Petri dishes to prevent drying and were incubated for 1 h at 37°C. The slides were then flooded with a 1 : 10 dilution of Guinea-pig complement and incubated for an additional 30 min. By this time, plaques produced by antibody producing cells were visible. Due to size variations of plaques, the total number of plaques on the entire slide was quantified under slight magnification using a dissecting microscope.

Effect of GcMAF on immune development against Ehrlich ascites tumour was assessed by transplantability of the ascites tumour

The GcMAF (100 pg/mouse) was administered into the peritoneal cavity or subcutaneously lower dorsal followed by injection of heat-killed Ehrlich ascites tumour (10^6 cells) into the peritoneal cavity. At various intervals (2–10 days) after immunization with the heat-killed tumour cells, live Ehrlich ascites tumour (5×10^5 cells) was transplanted into the peritoneal cavity. Immune development against Ehrlich ascites tumour was assessed by growth or rejection of the transplanted Ehrlich ascites tumour as determined by live cell counts in the peritoneal cavity. For a control study without GcMAF pretreatment, mice were immunized with heat-killed Ehrlich ascites tumour and live Ehrlich ascites tumour was transplanted into the peritoneal cavity.

Results

Freund's incomplete adjuvant induces inflammation that activates macrophages

Freund's incomplete adjuvant (1 μg/mouse) was injected into the peritoneal cavity or lower dorsal of mice. Five days later, peritoneal macrophages were harvested and assayed for macrophage activation and cell counts. As shown in Table 1, peritoneal macrophages were greatly activated as measured by 10-fold and 8.5-fold increased superoxide generating capacity in mice that received peritoneal cavity and lower dorsal subcutaneous injection of the adjuvant, respectively. This macrophage activation is a result of the inflammatory effect of the adjuvant. The cell counts of peritoneal macrophages in mice that received adjuvant in the peritoneal cavity and lower dorsal increased 22-fold and 15-fold, respectively. Although some chemotactic effect of inflammation was found, the increased cell counts and activation of peritoneal macrophages were not affected greatly by the administration sites of the adjuvant. These results suggest that inflammation-derived macrophage activating factor acts on the macrophage progenitor cells for both mitogenesis and activation, as we have previously shown that macrophage activating factor acts on the progenitor cells for mitogenesis.[21,27,28] A large number of the activated macrophages from the progenitor cells were distributed systemically.

Effect of administration sites of Freund's complete adjuvant on macrophage activation

Freund's complete adjuvant (1 μg/mouse) was also injected into the peritoneal cavity and lower dorsal of mice. Two days after administration of the adjuvant, peritoneal macrophages were harvested and assayed for macrophage cell counts and activation. As shown in Table 2, peritoneal macrophage cell counts increased to ~68-fold in peritoneally treated mice and 20-fold in lower dorsal-treated mice. Because of severe inflammation induced by Freund's complete adjuvant, a chemotactic effect was evident. Activation of peritoneal macrophages as measured by superoxide generating capacity increased ~15-fold in peritoneally

Table 1 Peritoneal macrophage activation and cell counts 5 days after administration of Freund's incomplete adjuvant to peritoneal cavity or lower dorsal of BALB/c mice

Site of administration 5 days post-administration	Macrophage counts (Peritoneal fluid/mL)	Superoxide generation (nmol of superoxide/10^6 cells)
Untreated	1.60×10^6	0.29 ± 0.02
Peritoneal	3.54×10^7	2.93 ± 0.08
Lower dorsal	2.39×10^7	2.45 ± 0.07

Values of superoxide generation represent mean ± SEM of triplicate assays.

treated mice and 13-fold in lower dorsal-treated mice. It seems obvious that in peritoneally treated mice the inflammation-derived macrophage activating factor activates local resident peritoneal macrophages before interacting with the progenitor cells. Thus, macrophages of peritoneally treated mice are slightly more activated than those of lower dorsal-treated mice.

At 4 days after administration of Freund's complete adjuvant, peritoneal macrophage cell counts increased to ~75-fold in peritoneally treated mice and 58-fold in lower dorsal-treated mice as shown in Table 2. Activation of peritoneal macrophages as measured by superoxide generating capacity increased ~22-fold in both peritoneally and lower dorsal-treated mice. Thus, Freund's complete adjuvant induces severe inflammation that generates a large amount of macrophage activating factor that stimulates the macrophage progenitor cells for mitogenesis and activation.

Effect of administration sites of the enzymatically generated macrophage activating factor, GcMAF, on activation of peritoneal macrophages

The enzymatically generated macrophage activating factor, GcMAF, should substitute for the adjuvants. A minute amount (20 pg/day) of GcMAF was injected once or twice into the peritoneal cavity. Two days after the first GcMAF administration, peritoneal macrophages were harvested and assayed for macrophage cell counts and activation. As shown in Table 3, macrophage cell counts increased ~10-fold and 20-fold in mice that received one and two GcMAF injections, respectively. An ~20-fold increased superoxide generating capacity was observed in both the once- or twice-GcMAF-treated groups. When GcMAF was administered once or twice into the lower dorsal, peritoneal macrophage cell counts increased to only 1.3- and 2.2-fold, and superoxide generating capacity increased ~10- and 11-fold, respectively. Thus, a chemotactic effect on macrophages was evident in peritoneally treated mice. The GcMAF activates the resident peritoneal macrophages before interacting with the progenitor cells. Because a large number of macrophages were recruited to the peritoneal cavity where GcMAF (non-inflammatory protein) was administered, it is possible that the activated resident peritoneal macrophages secrete a chemotactic factor, or GcMAF itself has a chemotactic function.

At 4 days after three daily injections of GcMAF (20 pg/day), peritoneal macrophage cell counts increased ~160-fold in peritoneally treated mice and 10-fold in lower dorsal-treated mice as shown in Table 3. An ~37-fold increased superoxide generating capacity was observed in peritoneally treated mice while a 29-fold increased super-

Table 2 Peritoneal macrophage activation and cell counts 2 and 4 days after administration of Freund's complete adjuvant to peritoneal cavity or lower dorsal of BALB/c mice

Time and site of administration	Macrophage counts (Peritoneal fluid/mL)	Superoxide generation (nmol of superoxide/10^6 cells)
2 Days post-administration		
Untreated	1.77×10^6	0.29 ± 0.03
Peritoneal	1.21×10^8	4.25 ± 0.11
Lower dorsal	3.48×10^7	3.80 ± 0.07
4 Days post-administration		
Untreated	1.75×10^6	0.31 ± 0.01
Peritoneal	1.31×10^8	6.81 ± 0.12
Lower dorsal	1.02×10^8	6.69 ± 0.13

Values of superoxide generation represent mean ± SEM of triplicate assays.

Table 3 Peritoneal macrophage activation and cell counts after daily administration of macrophage activating factor (20 pg/mouse) to peritoneal cavity or lower dorsal of BALB/c mice

Site of administration Days/number of injection	Macrophage counts (Peritoneal fluid/mL)	Superoxide generation (nmol of superoxide/10^6 cells)
2 Days post-administration		
Untreated	2.37×10^6	0.37 ± 0.04
Peritoneal (1 injection)	2.31×10^7	7.41 ± 0.13
Peritoneal (2 injection)	4.71×10^7	7.63 ± 0.16
Lower dorsal (1 injection)	3.14×10^6	3.79 ± 0.10
Lower dorsal (2 injection)	5.26×10^6	4.23 ± 0.08
4 Days post-administration		
Untreated	1.36×10^6	0.31 ± 0.01
Peritoneal (3 injection)	2.17×10^8	11.45 ± 0.32
Lower dorsal (3 injection)	1.31×10^7	8.99 ± 0.18

Values of superoxide generation represent mean ± SEM of triplicate assays.

oxide generating capacity was observed in lower dorsal-treated mice.

Two days after administration of a single larger dose (100 pg/mouse) of GcMAF into peritoneal cavity and lower dorsal, peritoneal macrophages were harvested and assayed for cell counts and activation. As shown in Table 4, peritoneal macrophage cell counts increased ~97-fold in peritoneally treated mice and 20-fold in lower dorsal-treated mice. An ~25-fold increased superoxide generating capacity in peritoneally treated mice and 22.5-fold increased superoxide generating capacity in lower dorsal-treated mice were observed. Therefore, macrophages were recruited to the peritoneal cavity where GcMAF (non-inflammatory protein) was administered, supporting the hypothesis that GcMAF plays a chemotactic role directly or indirectly.

At 4 days after one injection of GcMAF (100 pg/mouse), peritoneal macrophage cell counts increased ~180-fold in peritoneally treated mice and 40-fold in lower dorsal-treated mice. The superoxide generating capacity of macrophages increased ~39-fold in peritoneally treated mice and 33-fold in lower dorsal-treated mice (Table 4).

Rapid increase of the number of antibody secreting cells in mice after administration of GcMAF and sheep erythrocytes

Mice were intraperitoneally inoculated with SRBC 6 h after administration of 100 pg GcMAF to the peritoneal cavity or lower dorsal subcutaneous. At various intervals (1–5 days) after immunization, IgM antibody secreting cells in the spleen were determined using the Jerne plaque assay.[26] As shown in Table 5, in peritoneally GcMAF-treated mice, a day after administration of SRBC, the number of antibody secreting splenic cells was beginning to increase. Two days after immunization, the number of antibody secreting cells had increased to 10.22×10^4 antibody secreting cells (PFC)/spleen. By the fourth day, the number of antibody secreting cells reached the maximal level ($\sim38.23\times10^4$ PFC/spleen). Thereafter, the number of antibody-secreting cells in the spleen gradually decreased. In contrast, mice that received an injection of SRBC alone produced a smaller number ($\sim3.77\times10^4$ PFC/spleen) of antibody secreting cells 4 days after SRBC injection.

In lower dorsal GcMAF treated mice, a day after administration of SRBC, the number of antibody secreting cells was also beginning to increase. Two days after immunization, the number of antibody secreting cells had increased to 4.21×10^4 PFC/spleen. By the fourth day the number of antibody secreting cells reached the maximum level ($\sim27.38\times10^4$ PFC/spleen). Thereafter, the number of antibody secreting cells in the spleen gradually decreased.

GcMAF as an adjuvant for immune development against Ehrlich ascites tumour

The GcMAF (100 pg/mouse) was administered to the peritoneal cavity or lower dorsal followed by injection of

Table 4 Peritoneal macrophage activation and cell counts 2 and 4 days after single administration of macrophage activating factor (100 pg/mouse) to peritoneal cavity or lower dorsal of BALB/c mice

Site of administration Days/number of injection	Macrophage counts (Peritoneal fluid/mL)	Superoxide generation (nmol of superoxide/10^6 cells)
2 Days post-administration		
Untreated	1.14×10^6	0.37 ± 0.04
Peritoneal	1.09×10^8	9.14 ± 0.19
Lower dorsal	2.27×10^7	8.31 ± 0.13
4 Days post-administration		
Untreated	1.23×10^6	0.30 ± 0.05
Peritoneal	2.21×10^8	11.72 ± 0.22
Lower dorsal	4.87×10^7	9.88 ± 0.15

Values of superoxide generation represent mean ± SEM of triplicate assays.

Table 5 Time–course studies on development of cells secreting antibody against sheep erythrocytes (SRBC) in BALB/c mice immunized with SRBC after administration of macrophage activating factor (GcMAF) (100 pg/mouse) to peritoneal cavity or lower dorsal of BALB/c mice

After SRBC immunization (Days)	Antibody secreting cells/spleen ($\times10^4$)		
	SRBC only	$GcMAF_{pc}$ + SRBC	$GcMAF_{ld}$ + SRBC
1	0.06 ± 0.02	1.89 ± 0.19	1.03 ± 0.16
2	0.62 ± 0.09	10.22 ± 1.51	4.21 ± 1.18
3	1.13 ± 0.23	22.58 ± 1.89	11.87 ± 2.23
4	3.77 ± 0.65	38.23 ± 6.21	27.38 ± 4.44
5	2.57 ± 0.49	30.28 ± 3.05	21.28 ± 3.33

BALB/c mice were intraperitoneally inoculated with SRBC 6 h after administration of 100 pg GcMAF/mouse into peritoneal cavity ($GcMAF_{pc}$) or lower dorsal ($GcMAF_{ld}$). At various intervals (1–5 days) after immunization, IgM antibody secreting cells in the spleen were determined using the Jerne plaque assay. The number of plaques (IgM secreting cells) was quantified microscopically on various days post-SRBC injection. The number of plaque-forming cells (PFC) per spleen is expressed as the mean value of triplicate assays ± SEM.

heat-killed Ehrlich ascites tumour (10^6 cells) into the peritoneal cavity. At various intervals (2–10 days) after GcMAF-primed immunization of mice with the killed ascites tumour cells, live Ehrlich ascites tumour (5×10^5 cells) was transplanted into the peritoneal cavity and tumour transplantability was tested. As shown in Table 6, 4 days after GcMAF administration to the peritoneal cavity and immunization with heat-killed tumour, Ehrlich ascites tumour is no longer transplantable in these mice, as demonstrated by an undetectable level of viable cell counts ($< 5.0\times10^3$ cells/mL). In contrast, 4 days after GcMAF administration to the lower dorsal and immunization with heat-killed tumour, transplanted live tumour cells were still detectable but decreased to 2.2×10^4 cells/mL, while 6 days after the GcMAF-primed immunization the ascites tumour was not transplantable. For the control study without GcMAF pretreatment, immunization of mice with heat-killed Ehrlich ascites tumour did not protect mice from growth of transplanted tumour for at least 10 days after immunization.

Discussion

Adjuvants induce inflammation.[1,3,4] In particular, adjuvants that produce severe inflammation augment development of antibodies against poorly immunogenic antigens (e.g. purified or cloned microbial components and tumour antigens). Inflamed tissues release lysophospholipids that stimulate lymphocytes to develop macrophage activating factor.[5–7] Thus, the extent of adjuvant inflammatory effects can be assessed by the degree of macrophage activation. Potent inflammatory adjuvants induce extensive activation of macrophages. Lysophospholipids also have a chemotactic function for recruiting macrophages. Thus, adjuvants induce inflammation that results in macrophage activation and chemotaxis. Because macrophage activation for phagocytosis and antigen processing is the first step of immune development, adjuvant-primed macrophage activating factor plays a key role in antibody production. Therefore, macrophage activating factor can act as a non-inflammatory adjuvant. The GcMAF is probably the most potent macrophage activating factor ever discovered.[21,29] Enzymatically generated macrophage activing factor not only activates local resident macrophages but also is mitogenic to the progenitor cells for production of a large number of macrophages.[27,28] Thus, a large number of the activated macrophages derived from the GcMAF-stimulated progenitor cells can be distributed systemically. However, some chemotaxis of the macrophages was observed at the site where GcMAF was administered. Nevertheless, peritoneal macrophages of mice treated with a minute amount (20 or 100 pg/mouse) of GcMAF are highly activated as measured by a 30- to 40-fold increased superoxide generating capacity irrespective of GcMAF administration sites (either peritoneal or lower dorsal). In spite of an enormous increase in macrophage cell counts, the level of peritoneal macrophage activation per cell in peritoneally treated mice is not greatly different from those of peritoneal macrophages in lower dorsal-treated mice. Thus, GcMAF acts on the macrophage progenitor cells for both activation and mitogenesis. Unlike Freund's adjuvant, GcMAF is dispersal. However, because GcMAF has a chemotactic effect on macrophages, GcMAF is a most effective adjuvant if GcMAF and antigens are administered at the same site. In particular, a few days after GcMAF administration, antigens should be injected to the GcMAF administration site where the activated macrophages are being recruited for phagocytosis and antigen processing. Because the potencies of the macrophage activating factor and its mitogenic and chemotactic activities regulate the level of immune response and antibody production, GcMAF should serve as a potent adjuvant for a variety of antigens. A large number of the activated macrophages can rapidly phagocytize and process the antigens.[21] The processed antigens are immediately transmitted to T and B cells for production of antibodies. We observed that administration of GcMAF and immunogens such as SRBC to mice produced a large number of antibody secreting cells beginning a day after immunization.

Table 6 Immune development in BALB/c mice at various periods after macrophage activating factor (GcMAF)-primed immunization with killed Ehrlich ascites tumour is assessed by transplantability of the ascites tumour

Days Time of tumour transplant	Growth of transplanted live ascites tumour (5×10^5 cells) after GcMAF-primed immunization with heat-killed tumour		
	Live tumour only	Live tumour/$GcMAF_{pc}$-imm	Live tumour/$GcMAF_{ld}$-imm
2	+ (1.16×10^7)	± (3.54×10^5)	+ (1.11×10^6)
4	+ (3.34×10^6)	± (1.21×10^4)	± (2.64×10^4)
6	+ (2.34×10^6)	− ($< 5.0\times10^3$)	± (2.21×10^4)
8	+ (1.48×10^6)	− ($< 5.0\times10^3$)	− ($< 5.0\times10^3$)
10	+ (1.01×10^6)	− ($< 5.0\times10^3$)	− ($< 5.0\times10^3$)

Control (no immunization): 5×10^5 cells increased to 1.11×10^8 cells in 14 days

BALB/c mice were intraperitoneally immunized with 10^6 heat-killed Ehrlich ascites tumour 6 h after administration of 100 pg GcMAF/mouse into the peritoneal cavity ($GcMAF_{pc}$-imm) or lower dorsal ($GcMAF_{ld}$-imm). At various intervals (2–10 days) after immunization, live Ehrlich ascites tumour was transplanted in peritoneal cavity. Two weeks later, growth (+), reduced growth (±) or rejection (−) of tumour transplant were determined by viable cell counts of ascites tumour in the peritoneal cavity. PBS (8 mL) was injected into the peritoneal cavity and the peritoneal lavage was aspirated. The number of live ascites cells was quantified microscopically on 14 days after GcMAF-primed immunization. The number of viable tumour cells/mL in the peritoneal cavity is expressed as the mean value of triplicate assays ± SEM.

Although malignant tumours are poorly immunogenic, many breast cancer patients often develop antibodies against breast tumour antigen.[30–33] Mice immunized with killed Ehrlich ascites tumour (murine mammary adenocarcinoma) develop immunity against the tumour.[29] The GcMAF-primed immunization of mice with heat-killed Ehrlich ascites tumour cells allows the mice to rapidly develop immunity against the ascites tumour. This was demonstrated by rejection of transplanted tumour a few days after GcMAF-primed immunization. Since the tumoricidal capacity of macrophages is observed preferentially via the IgG (Fc receptor)-mediated pathway,[34–36] GcMAF-primed immunization of mice with heat-killed Ehrlich ascites tumour cells seems likely to induce development of antibodies.

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