Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8539–8543, October 1991
Immunology

Vitamin D_3 binding protein (group-specific component) is a precursor for the macrophage-activating signal factor from lysophosphatidylcholine-treated lymphocytes

(inflammation/B and T cells/phagocytosis/glycoprotein/glycosidases)

NOBUTO YAMAMOTO*†‡ AND SADAMU HOMMA†§

*Department of Biochemistry, Temple University School of Medicine, 3420 North Broad Street, Philadelphia, PA 19140; and †Department of Microbiology and Immunology, Hahnemann University School of Medicine, Philadelphia, PA 19102

Communicated by Sidney Weinhouse, May 31, 1991

ABSTRACT **A brief (30 min) treatment of mouse peritoneal cells (mixture of nonadherent lymphocytes and adherent macrophages) with 1–20 μg of lysophosphatidylcholine (lyso-PC) per ml in serum-supplemented RPMI medium 1640, followed by a 3-hr cultivation of the adherent cells alone, results in a greatly enhanced Fc receptor-mediated phagocytic activity of macrophages. This rapid process of macrophage activation was found to require a serum factor, the vitamin D_3 binding protein (the human protein is known as group-specific component; Gc). Efficient activation of macrophages was achieved by using medium containing purified human Gc protein. Analysis of intercellular signal transmission among nonadherent (B and T) cells revealed that lyso-PC-treated B cells modify Gc protein to yield a proactivating factor, which can be converted by T cells to the macrophage-activating factor. This rapid generation process of the macrophage-activating factor was also demonstrated by stepwise incubation of Gc protein with lyso-PC-treated B-cell ghosts and untreated T-cell ghosts, suggesting that Gc protein is modified by preexisting membranous enzymes to yield the macrophage-activating factor. Incubation of Gc protein with a mixture of β-galactosidase and sialidase efficiently generated the macrophage-activating factor. Stepwise incubation of Gc protein with B- or T-cell ghosts and sialidase or β-galactosidase revealed that Gc protein is modified by β-galactosidase of B cells and sialidase of T cells to yield the macrophage-activating factor. Administration to mice of a minute amount (4–10 pg per mouse) of *in vitro*, enzymatically generated macrophage-activating factor resulted in a greatly enhanced (3- to 7-fold) ingestion activity of macrophages.**

Microbial infections of various tissues lead to inflammation, which results in chemotaxis and activation of phagocytes. Inflamed tissues release lysophospholipids as a consequence of cellular damage and resultant activation of phospholipase A (1, 2). Inflamed cancerous tissues produce alkyllysophospholipids and alkylglycerols as well as lysophospholipids (2–4) because cancerous cell membranes contain alkylphospholipids and monoalkyldiacylglycerols (5, 6). These lysophospholipids and alkylglycerols are potent macrophage-activating agents (3, 4, 7). Administration of lysophospholipids (5–20 μg per mouse) and alkylglycerols (10–100 ng per mouse) to mice activates macrophages to phagocytize target cells via the Fc receptor but not via the C3b receptor (1–4, 7, 8) and to generate superoxide (4). *In vitro* treatment of mouse peritoneal adherent cells (macrophages) alone with lysophosphatidylcholine (lyso-PC) or dodecylglycerol (DDG) results in no enhanced ingestion activity of macrophages (2–4, 7–9). However, incubation of peritoneal cells (mixture of adherent and nonadherent cells) with lyso-PC or DDG for 2–3 hr markedly enhanced Fc receptor-mediated phagocytic activity of macrophages, implying a participation of nonadherent cells to the activation of macrophages (2–4, 7–9). Thus, *in vivo* activation of macrophages by lysophospholipids and alkylglycerols can be reproduced *in vitro* (2–4, 7–9); inflammation metabolites of both normal and cancerous membrane lipids, lysophospholipids and alkylglycerols, can induce the same mechanism of macrophage activation.

Since participation of nonadherent (B and T) cells is essential for the macrophage activation (2–4, 7, 8), we proposed that there is a signal transmission from lyso-PC- or DDG-treated nonadherent cells to adherent cells. This intercellular signal transmission is a rapid process, which can occur within 30 min (3, 7–9). Analysis of macrophage-activating signal transmission among the nonadherent cells revealed that lyso-PC- or DDG-treated B cells can generate and transmit a macrophage-proactivating signal factor to untreated (or treated) T cells, which in turn generate a macrophage-activating factor (7–9). To identify the chemical nature of the signal factor(s) transmitted between lymphocytes, a serum-free medium was required. When 0.1% egg albumin was substituted for 10% fetal calf serum (FCS) in the culture medium, phagocytic activity of macrophages was greatly reduced (9–11). Therefore, we assume that there might be a contributory role of serum factor(s) necessary for the activation of macrophages.

Since the intercellular signal transmission is extremely rapid, the serum factor could be modified by preexisting (rather than inducible) functions of B and T cells to yield the macrophage-activating factor (9, 10). This hypothesis is confirmed by the demonstration that a stepwise incubation of DDG-treated B-cell ghosts and untreated T-cell ghosts with FCS generates the macrophage-activating factor (9). Electrophoretic fractionation of FCS showed that the α_2-globulin fraction is required for lyso-PC- and DDG-primed macrophage activation (10, 11). The major components of α_2-globulin are vitamin D_3 binding protein (the human protein is known as group-specific component; Gc protein), α_2-macroglobulin, haptoglobin, and α_2-HS-glycoprotein. By the use of antibodies against human α_2-globulin components and a monoclonal antibody against Gc protein along with chromatographic fractionation of human serum, we demonstrated that Gc protein is a serum factor required for lyso-PC-primed activation of macrophages (11).

In the present study, we present evidence that vitamin D_3 binding protein is a precursor for the macrophage-activating

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Abbreviations: Gc protein, human vitamin D_3 binding protein (group-specific component); lyso-PC, lysophosphatidylcholine; DDG, dodecylglycerol; FCS, fetal calf serum.

‡To whom reprint requests should be addressed.
§Permanent address: 1st Department of Internal Medicine, The Jikei University School of Medicine, Minato-ku, Tokyo, Japan.

factor, that human vitamin D_3 binding protein, Gc, is converted by the membranous glycosidases of B and T cells to the macrophage-activating factor, and that enzymatic conversion of Gc protein to the macrophage-activating factor can occur *in vitro*.

MATERIALS AND METHODS

Animals. Female BALB/c mice, 7–12 weeks of age, were obtained from the Jackson Laboratory. Mice were fed Purina Mouse Chow and water ad libitum.

Chemicals and Reagents. Lyso-PC was purchased from Sigma. Phosphate-buffered saline (PBS; 0.01 M sodium phosphate/0.9% NaCl) was prepared without addition of Ca^{2+} and Mg^{2+} ions. Human Gc protein consists of three genetic groups (protein polymorphisms): Gc1f, Gc1s, and Gc2. A mixture of Gc1f and Gc1s (Gc1) was kindly supplied by J. G. Haddad and M. A. Kowalski (University of Pennsylvania), who purified it by actin affinity chromatography from pooled blood bank plasma. Gc proteins were also purified in our laboratory by the immunosorbent method of Kimura *et al.* (12) with anti-Gc immobilized with Sepharose 4B. Glycosidases were purchased from Boehringer Mannheim. Using the *Limulus* amebocyte lysate assay (2), we routinely tested for lipopolysaccharide contamination in the stock solution of lyso-PC, stock solutions of human Gc protein, and culture media.

Culture Media. For *in vitro* manipulation and cultivation of nonadherent and adherent cells, 10% FCS-supplemented RPMI medium 1640 (FCS medium) and 0.1% egg albumin-supplemented RPMI medium 1640 (EA medium) were used.

***In Vitro* Treatment of Peritoneal Cells.** The detailed procedure for harvesting, cultivation, and enumeration of peritoneal adherent cells (macrophages) and nonadherent cells has been described (3, 7). Briefly, cells were harvested by injecting into the peritoneum 6–8 ml of ice-cold PBS (pH 7.1) containing 5–10 units of heparin per ml with a tuberculin needle attached to a 10-ml syringe. The abdomen was massaged for several seconds, and the peritoneal fluid was then withdrawn with a 21-gauge needle attached to a 10-ml syringe. The cells were then washed three times with cold PBS without heparin and resuspended in EA medium. The peritoneal cells (mixture of adherent and nonadherent cells) were placed in 16-mm plastic culture wells (containing 12-mm cover glasses) and incubated at 37°C in a humidified 5% CO_2/95% air incubator for 30 min to allow macrophage adherence to cover glasses. These peritoneal cell cultures were incubated with 1 μg of lyso-PC per ml in EA medium at 37°C. After a 30-min incubation, the nonadherent cells were washed three times with PBS. The lyso-PC-treated nonadherent cells were added to untreated adherent cells. The mixture of these cells was cultured in EA medium or EA medium supplemented with purified Gc protein for 3 hr prior to the phagocytosis assay.

Phagocytosis Assay. Phagocytosis of sheep erythrocytes (E) coated with IgG of anti-E (hemagglutinin), as Fc receptor-mediated phagocytosis, was described previously (7, 9). The results are expressed as the ingestion index as described by Bianco *et al.* (13). Ingestion index = (the percentage of macrophages with phagocytized E) × (the average number of E phagocytized per ingested macrophage).

Stimulation of Splenic Lymphocytes and Stepwise Preparation of Conditioned Media. Collection of mouse splenic cells and fractionation of B and T cells have been described (7). For the preparation of conditioned medium, B cells were treated with 1 μg of lyso-PC per ml in EA medium for 30 min, washed with PBS, resuspended in EA medium supplemented with various concentrations of Gc1 (0.26–2600 ng/ml), and cultured for 2 hr. The culture medium was further used as a medium for culturing untreated T cells for 2 hr. The resultant conditioned medium was then added to untreated peritoneal adherent cells (macrophages) and incubated for 3 hr prior to the ingestion assay. It should be noted here that 26 μg of Gc per ml is equivalent to the concentration of Gc protein in FCS medium.

Preparation of Splenic Nonadherent Cell Ghosts. The procedure for preparation of splenic nonadherent cell ghosts (10) was used for preparation of splenic B- and T-cell ghosts.

Treatment of Gc Protein with Glycosidases. Purified Gc protein (2.6 μg of Gc1 or Gc2) in 1 ml of PBS (pH 5.5) containing 1 mM $MgSO_4$ was treated with 2 μl of the same buffer containing various glycosidases (0.1 unit) or their combination at 37°C for 1 hr.

***In Vivo* Assay of Enzymatically Generated Macrophage-Activating Factor.** The enzymatically modified Gc protein (40, 10, 4, and 1 pg) was administered intramuscularly to BALB/c mice weighing about 20 g. At 18 hr postadministration, peritoneal cells were collected and placed on 12-mm cover glasses in the 16-mm wells of tissue culture plates. The plates were incubated at 37°C for 30 min to allow adherence of macrophages. Phagocytic activity of macrophages was analyzed as described (7, 9).

RESULTS

Dose Effect of Purified Gc Protein on Macrophage Activation: Demonstration of Macrophage Activation by Stepwise Preparation of Conditioned Media of Lyso-PC-Treated B Cells and Untreated T Cells in the Presence of Gc1 Protein. We demonstrated that Gc protein is required for lyso-PC-primed activation of macrophages by using chromatographic fractionation of human serum and an antibody against Gc protein (11). Lyso-PC-treated B cells were cultured for 2 hr in EA medium containing various concentrations of purified Gc1 protein. These B-cell culture media were used to culture untreated T cells for an additional 2 hr. The resultant conditioned media were used for a 3-hr cultivation of macrophages. As shown in Fig. 1, at a Gc1 concentration of 26 ng/ml a greatly enhanced ingestion activity was observed. We have previously shown that stepwise modification of a serum factor by B and T cells yields a macrophage-activating factor (7–10). Thus, Gc protein appears to be a precursor for the macrophage-activating factor. As the concentration of Gc1 protein increased over 100 ng/ml, however, the level of ingestion activity decreased. At Gc1 concentrations >1000

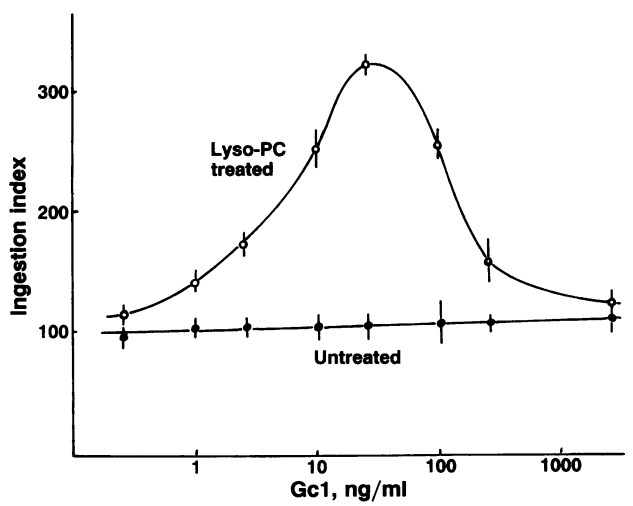

FIG. 1. Dose–response of purified Gc protein on *in vitro* activation of mouse macrophages. Stepwise-conditioned media of lyso-PC-treated B cells and untreated T cells in media containing various concentrations of Gc1 protein (0.26–2600 ng/ml) were prepared. The data are the mean ± SEM of triplicate assays.

ng/ml, no enhanced ingestion activity was observed. We propose that higher doses of Gc1 protein yield correspondingly larger amounts of the macrophage-activating factor but that an excess inhibits this macrophage response. To test this possibility, conditioned medium was prepared by stepwise cultivation of lyso-PC-treated B cells and untreated T cells in EA medium containing 2.6 μg of Gc1 per ml. Although there was no enhanced ingestion activity at a Gc1 concentration of 2.6 μg/ml, 30- and 100-fold dilution of this conditioned medium exhibited a greatly enhanced ingestion activity (Fig. 2).

Macrophage Activation by Conditioned Medium on Stepwise Incubation of Lyso-PC-Treated B-Cell Ghosts and Untreated T-Cell Ghosts in EA Medium Containing Gc1 Protein. Since generation of macrophage-activating factor is a rapid process and the preexisting membranous functions of B and T cells can modify a serum factor to yield the macrophage-activating factor (9, 10), Gc proteins were tested as to whether incubation with lyso-PC-treated lymphocyte cell ghosts would generate the macrophage-activating factor. The B-cell ghosts (10^6/ml) were treated with 1 μg of lyso-PC per ml in EA medium for 30 min, washed with PBS, and incubated in EA medium containing 2.6 μg of Gc1 per ml for 2 hr. This conditioned medium was used in an incubation of untreated T-cell ghosts for 2 hr. The resultant conditioned medium, diluted 3000-fold, markedly activated macrophages as shown in Table 1. Therefore, we conclude that Gc1 protein is modified by preexisting membranous enzymes of B and T cells to yield a macrophage-activating factor. A similar stepwise preparation of conditioned medium of lyso-PC-treated T-cell ghosts and untreated B-cell ghosts with EA medium containing Gc1 protein (2.6 μg/ml) did not generate macrophage-activating factor. This observation indicates the requirement for lyso-PC treatment of B cells to activate the enzyme or to make the enzyme accessible to Gc glycoprotein.

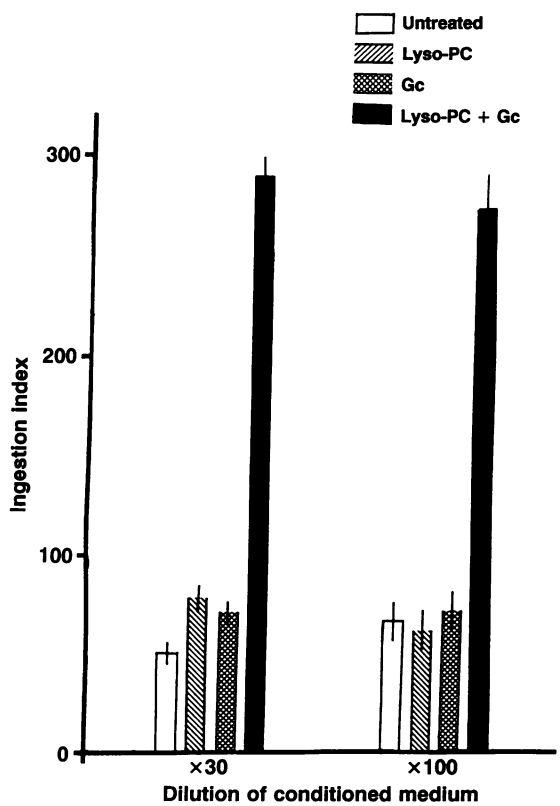

FIG. 2. Quantitative analysis of macrophage-activating factor contained in conditioned media prepared by stepwise transfer of EA medium containing purified Gc protein with lyso-PC-treated B cells and untreated T cells. Mouse splenic B cells (10^6/ml) were untreated or treated with 1 μg of lyso-PC per ml for 30 min at 37°C in EA medium. After washing in PBS, the untreated or treated B cells were incubated in EA medium in the presence or absence of purified Gc protein (2.6 μg/ml) for 2 hr. The cultured media were transferred to untreated splenic T cells (10^6/ml) and incubated for an additional 2 hr. The resultant conditioned media were diluted with EA medium and used for the macrophage activation assay. Data are mean ± SEM of triplicate assays.

Table 1. Activation of macrophages by stepwise-conditioned medium of lyso-PC-treated B-cell ghosts and untreated T-cell ghosts in EA medium containing Gc protein

Fold dilution of medium	Conditioned medium: Untreated − Gc1	Untreated B + Gc1	Lyso-PC-treated B + Gc1
300	92 ± 21	99 ± 21	61 ± 12
3000	81 ± 8	108 ± 10	318 ± 25

B-cell ghosts (10^6/ml) were treated with 1 μg of lyso-PC per ml in EA medium for 30 min. After washing with PBS, lyso-PC-treated B-cell ghosts were incubated in EA medium with or without 2.6 μg of Gc1 protein per ml for 2 hr. The resultant conditioned media were used for a 2-hr incubation of untreated T-cell ghosts (10^6/ml). The ultimate conditioned media were further diluted with EA medium for cultivation of peritoneal adherent cells for the macrophage ingestion assay. Values represent mean ± SEM of triplicate assays.

Modification of Gc1 Protein by Glycosidases. A preliminary Western immunoblotting analysis revealed no difference in relative molecular weights of the macrophage-activating factor and native Gc protein. Thus, membranous glycosidases but not proteases appeared to be candidates for modification of the Gc glycoprotein. Gc1 protein (2.6 μg/ml) was treated with various combinations of glycosidases, then diluted 10,000-fold, and added to cultured adherent macrophages for 2 hr. The Gc1 protein treated with a combination of sialidase (0.1 unit/ml) and β-galactosidase (0.1 unit/ml) efficiently activated macrophages, as shown in Table 2. Production of the macrophage-activating factor by treatment of purified human Gc1 protein with the two glycosidases indicates that removal of sialic acid and galactose residues of the oligosaccharide of Gc1 (mixture of Gc1f and Gc1s subtypes) results in formation of the macrophage-activating factor. Both Gc1f and Gc1s subtypes of Gc1 are known to carry sialic acid (14–16). Since stepwise modification of Gc1 by two nonadherent (B and T) cells is required for macrophage activation, we propose that the stepwise digestion of Gc1 glycoprotein by the two glycosidases (i.e., β-galactosidase and sialidase) of nonadherent cells yields the macrophage-activating factor.

Table 2. Activation of macrophages by glycosidase-treated Gc protein

Glycosidase	Ingestion index: Gc1	Gc2
None	48 ± 9	59 ± 13
Sialidase	38 ± 9	81 ± 9
β-Galactosidase	39 ± 6	175 ± 22
α-Mannosidase	40 ± 11	76 ± 15
Sialidase + β-galactosidase	174 ± 21	157 ± 34
Sialidase + α-mannosidase	51 ± 4	74 ± 15

After enzyme treatment, the reaction mixture was further diluted 10,000-fold with EA medium and used as a medium for a 3-hr cultivation of untreated peritoneal adherent cells for the macrophage phagocytosis assay. Reaction mixture without Gc protein showed no enhancement of phagocytic activity. Values represent mean ± SEM of triplicate assays.

Modification of Gc2 Protein by Glycosidase. Gc2 protein, when treated with β-galactosidase, was efficiently converted to the macrophage-activating factor as shown in Table 2, but treatment with sialidase had no effect on generation of the macrophage-activating factor. Therefore, removal of a galactose residue from Gc2 glycoprotein results in formation of the macrophage-activating factor.

Defining the Enzymatic Functions of Nonadherent (B and T) Cells for Conversion of Gc Protein to the Macrophage-Activating Factor. Since the nonadherent (B and T) cells convert Gc1 protein to the macrophage-activating factor, these cells should carry the two glycosidases (β-galactosidase and sialidase). The enzymatic function of each nonadherent (B or T) cell type was defined as follows. To identify the enzymatic function of T cells, we first prepared the conditioned medium of lyso-PC-treated B-cell ghosts with Gc1 protein in EA medium. This conditioned medium, containing a signal factor, termed proactivating factor, was treated with β-galactosidase or sialidase. The resultant medium was used for a 3-hr cultivation of adherent cells (macrophages) prior to the phagocytosis assay. As shown in experiment I of Table 3, sialidase but not β-galactosidase activated macrophages. Thus, this observation suggests that T cells carry the function corresponding to sialidase, which can convert the proactivating factor to the macrophage-activating factor. Indeed, it has been demonstrated that T cells carry sialidase in the membranes (17–19).

To identify the enzymatic function of B cells, Gc1 protein was first treated with β-galactosidase or sialidase and added to T-cell ghosts for a 2-hr incubation. The resultant conditioned media were used for a 3-hr cultivation of adherent cells. The conditioned medium of T-cell ghosts, when prepared with a medium containing β-galactosidase, but not sialidase-pretreated Gc1 protein, contained a high titer of the macrophage-activating factor (experiment II of Table 3). Therefore, it is concluded that the function of lyso-PC-treated B cells for macrophage activation is defined to be β-galactosidase.

Since treatment of Gc2 protein with β-galactosidase alone yields the macrophage-activating factor, either one of the nonadherent cell types (B or T cells) should be able to convert Gc2 protein to the macrophage-activating factor. Incubation of Gc2 protein with lyso-PC-treated B cells resulted in efficient formation of the macrophage-activating factor, whereas incubation of T cells with Gc2 protein produced no macrophage-activating factor. This result is in agreement with the fact that B cells carry β-galactosidase. To substantiate this conclusion, we studied the effect of Gc2 protein on activation of macrophages by the use of peritoneal cells of the SM/J mouse whose T cells are known to be deficient in sialidase (17–19). Lyso-PC-treated peritoneal cells of the SM/J mouse converted Gc2 protein, but not Gc1 protein, to the macrophage-activating factor.

Table 3. Defining the enzymatic functions of nonadherent (B and T) cells for conversion of Gc1 protein to the macrophage-activating factor

Exp.	Initial treatment	Second treatment	Ingestion index
I*	B-cell CM	β-Galactosidase	77 ± 10
	B-cell CM	Sialidase	303 ± 19
II†	β-Galactosidase	T-cell CM	325 ± 23
	Sialidase	T-cell CM	68 ± 12

CM, conditioned medium.

*Conditioned media of lyso-PC-treated B-cell ghosts were prepared with EA medium containing Gc1 protein. The resultant conditioned media were treated with β-galactosidase or sialidase (0.1 unit/ml) for 60 min at 37°C. The products were assayed for phagocytic activity.

†EA medium containing Gc1 protein was treated with β-galactosidase or sialidase (each at 0.1 unit/ml) for 60 min at 37°C. The resultant media were used for a 2-hr incubation of T-cell ghosts. The products were assayed for phagocytic activity. Values represent the mean ± SEM of triplicate assays.

Table 4. *In vivo* assay of the macrophage-activating factor generated by *in vitro* enzymatic modification of human Gc protein

Gc, pg per mouse	Ingestion index − glycosidases	Ingestion index + glycosidases
40	57 ± 16	322 ± 19
10	55 ± 10	353 ± 16
4	51 ± 12	163 ± 18
1	63 ± 18	114 ± 6

Gc1 protein (2.6 μg/ml) was treated with a mixture of β-galactosidase and sialidase (each at 0.1 unit/ml) for 60 min at 37°C. The resultant product was diluted and administered intramuscularly to BALB/c mice. At 18 hr postadministration, peritoneal cells were collected and assayed for phagocytic activity. Values represent mean ± SEM of triplicate assays.

***In Vivo* Assay of the *In Vitro* Generated Macrophage-Activating Factor.** When Gc1 proteins (2.6 μg/ml) were treated with a mixture of β-galactosidase and sialidase (0.1 unit/ml of each) for 60 min at 37°C, a highly active product as assayed by *in vitro* macrophage activation was obtained. When mice were inoculated with 10 pg (per mouse) of this *in vitro* enzymatically generated macrophage-activating factor, a 7-fold enhanced activation of peritoneal macrophages was observed (Table 4). Doses as low as 4 pg per mouse increased macrophage activation ≈3-fold.

DISCUSSION

A small amount of purified Gc protein, when incubated with lyso-PC-treated B-cell ghosts and T-cell ghosts, can be efficiently converted to the macrophage-activating factor. This observation implies that Gc protein is modified by the preexisting membranous enzymes of B and T cells to yield the macrophage-activating factor. The macrophage-activating factor can be generated by *in vitro* treatment of Gc protein with glycosidases. This serum-derived signaling factor, therefore, is distinct from the established interleukins. The formation of the macrophage-activating factor with lymphocytes is a very rapid process that can occur in a short period (30 min) (3, 7–9). Macrophages are activated to develop Fc receptor-mediated phagocytic activity in 2–3 hr after incubation with the macrophage-activating factor (3, 7–9). The sequence of proposed actions is depicted in Fig. 3*A*.

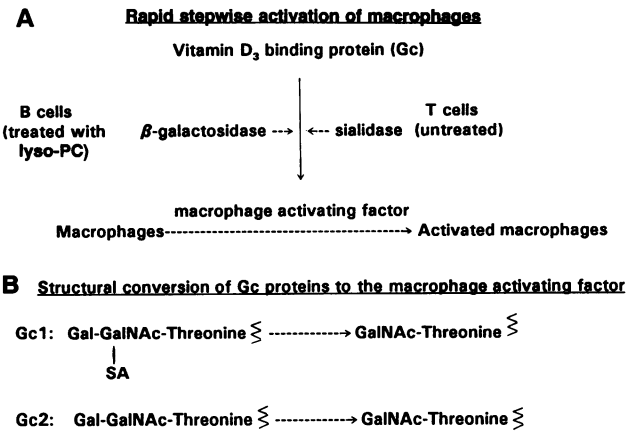

FIG. 3. Schematic illustration of macrophage activation and structural conversion of Gc protein to the macrophage-activating factor. SA, sialic acid.

Coppenhaver *et al.* (20) reported that sialic acid glycosylation of Gc1 molecules is a posttranslational process. Thus, sialic acid should be the outer terminal Gc1 oligosaccharide (15, 20). Viau *et al.* (15) suggested a linear terminal structure of the oligosaccharide: SA-Gal-GalNAc-threonine< (SA = sialic acid). Since the macrophage-activating factor was generated by stepwise modification of Gc1 protein by lyso-PC-treated B cells and untreated T cells, the glycosidases of B and T cells (β-galactosidase and sialidase, respectively) remove two saccharides in a stepwise fashion to yield the macrophage-activating factor (Fig. 3*A*). Since a stepwise preparation of the conditioned medium of untreated (or lyso-PC-treated) T cells and treated B cells does not generate macrophage-activating factor (7–9), the modification of Gc1 protein by B-cell function (i.e., β-galactosidase) should precede the modification by T-cell function, sialidase. Thus, a galactose residue should also be a terminal sugar (Gc1 in Fig. 3*B*). When the macrophage-activating factor in the conditioned medium was tested for absorption by various commercial lectin beads, *N*-acetylgalactosamine-specific lectins (21) removed the Gc1-derived macrophage-activating factor (unpublished results). Therefore, we propose that the oligosaccharide structure of the Gc1 protein carries a branched galactose and sialic acid termini at *N*-acetylgalactosamine as shown in Fig. 3*B*.

Since Gc2 protein can be converted to the macrophage-activating factor by treatment with β-galactosidase alone, we propose that the oligosaccharide terminal structure of Gc2 protein is Gal-GalNAc-threonine<. However, less than 1 in 10 Gc2 molecules is likely to be glycosylated, because the O-glycosylation of Gc2 protein is not demonstrated by the methods of Viau *et al.* (15) and Coppenhaver *et al.* (20). In fact <10% of Gc2 protein having faster mobility than the major Gc2 protein band can be seen in an electrophoretic pattern (16). We also propose that the saccharide terminal structure of the macrophage-activating factor derived from both Gc1 and Gc2 proteins appears to be GalNAc-threonine< (Fig. 3*B*).

In vitro enzymatic conversion of Gc protein to the macrophage-activating factor is so efficient that a high specific activity of the macrophage-activating factor is readily obtainable. This enzymatic conversion of Gc protein to the macrophage-activating factor can also be efficiently achieved by glycosidases immobilized on Sepharose beads (N.Y., unpublished results). Administration of a minute amount (about 10 pg per mouse) of the enzymatically generated macrophage-activating factor to mice results in a greatly enhanced phagocytic activity of the peritoneal macrophages at 18 hr (even in several hours) postadministration. Since glycosidases used for *in vitro* conversion of Gc protein to the macrophage-activating factor are the B- and T-cell functions required for production of the macrophage-activating factor, the enzymatic generation of the macrophage-activating factor bypasses the functions of B and T cells. Therefore, the enzymatically generated macrophage-activating factor may have an important role in the study and treatment of immunodeficient diseases.

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