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Immunotherapy of HIV-Infected Patients With Gc Protein-Derived Macrophage Activating Factor (GcMAF)

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Serum Gc protein (known as vitamin D_3-binding protein) is the precursor for the principal macrophage activating factor (MAF). The MAF precursor activity of serum Gc protein of HIV-infected patients was lost or reduced because Gc protein is deglycosylated by α-*N*-acetylgalactosaminidase (Nagalase) secreted from HIV-infected cells. Therefore, macrophages of HIV-infected patients having deglycosylated Gc protein cannot be activated, leading to immunosuppression. Since Nagalase is the intrinsic component of the envelope protein gp120, serum Nagalase activity is the sum of enzyme activities carried by both HIV virions and envelope proteins. These Nagalase carriers were already complexed with anti-HIV immunoglobulin G (IgG) but retained Nagalase activity that is required for infectivity. Stepwise treatment of purified Gc protein with immobilized β-galactosidase and sialidase generated the most potent macrophage activating factor (termed GcMAF), which produces no side effects in humans. Macrophages activated by administration of 100 ng GcMAF develop a large amount of Fc-receptors as well as an enormous variation of receptors that recognize IgG-bound and unbound HIV virions. Since latently HIV-infected cells are unstable and constantly release HIV virions, the activated macrophages rapidly intercept the released HIV virions to prevent reinfection resulting in exhaustion of infected cells. After less than 18 weekly administrations of 100 ng GcMAF for nonanemic patients, they exhibited low serum Nagalase activities equivalent to healthy controls, indicating eradication of HIV-infection, which was also confirmed by no infectious center formation by provirus inducing agent-treated patient PBMCs. No recurrence occurred and their healthy CD + cell counts were maintained for 7 years. ***J. Med. Virol. 81:16–26, 2009.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: macrophages; macrophage activation; eradication of HIV; immunosuppression

INTRODUCTION

Human immunodeficiency virus (HIV) can lead to acquired immunodeficiency syndrome (AIDS) that is characterized by opportunistic infections and by "opportunistic neoplasms" (e.g., Kaposi's sarcoma) as an evidence of immunosuppression [Dalgleish et al., 1984]. Impaired phagocytosis and bacteriocidal process of the phagocytes of AIDS patients have been reported from a number of laboratories [Fauci, 1984; Bender et al., 1985; Estevez et al., 1986; Roux-Lombard et al., 1986; Moller et al., 1990; Pos et al., 1992]. Macrophages are the major phagocytic and antigen presenting cells. Since macrophage activation for phagocytosis and antigen-presentation to B and T lymphocytes is the first indispensable step in development of both humoral and cellular immunity [Yamamoto et al., 1995; Yamamoto and Naraparaju, 1998], lack of macrophage activation leads to immunosuppression. In fact, immunosuppression in AIDS [Yamamoto et al., 1995; Yamamoto, 2006] and advanced cancer [Yamamoto et al., 1996, 1997] patients is caused by lack of macrophage activation.

Microbial infection induces inflammation that results in macrophage activation as for innate defense. The

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inflammation-derived macrophage activation is the process for generation of the principal macrophage activating factor [Yamamoto and Ngwenya, 1987; Ngwenya and Yamamoto, 1990; Yamamoto and Homma, 1991]. Administration of an inflamed tissue lipid metabolite, lysophosphatidylcholine (lyso-Pc) or other lysophospholipids, to mice rapidly activates macrophages for greatly enhanced phagocytic and superoxide generating capacities [Yamamoto and Ngwenya, 1987; Homma and Yamamoto, 1990; Ngwenya and Yamamoto, 1990; Yamamoto et al., 1994]. Inflammation-derived macrophage activation process requires serum Gc protein (known as vitamin D_3-binding protein) [Yamamoto and Homma, 1991; Yamamoto et al., 1991; Yamamoto, 1993, 1996] and participation of B and T lymphocytes [Ngwenya and Yamamoto, 1990; Yamamoto and Homma, 1991; Yamamoto, 1996]. Gc protein carries one trisaccharide composed of N-acetylgalactosamine with dibranched galactose and sialic acid termini at 420 threonine residue [Yamamoto and Homma, 1991; Yamamoto, 1993, 1996; Yamamoto and Kumashiro, 1993]. Stepwise hydrolysis of Gc protein with the inducible membranous β-galactosidase (Bgl_i) of inflammation-primed (or lyso-Pc-treated) B lymphocytes and the *Neu-1* sialidase of T lymphocytes yields the macrophage activating factor (MAF) [Yamamoto and Homma, 1991; Yamamoto, 1993, 1998a; Naraparaju and Yamamoto, 1994], the protein with N-acetylgalactosamine as the remaining sugar (Fig. 1a). Thus, Gc protein is the precursor for the principal MAF [Yamamoto and Homma, 1991; Yamamoto, 1996, 1998a].

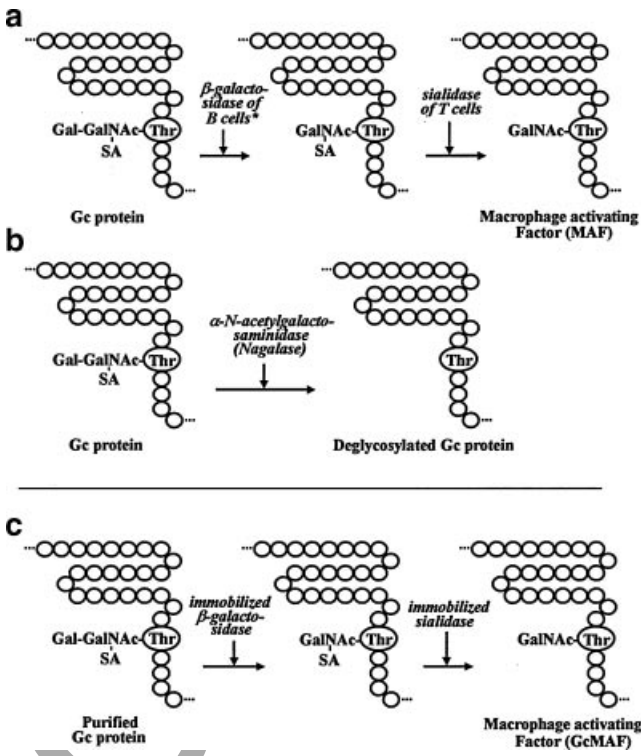

Fig. 1. Schematic illustration of formation of macrophage activating factor (**a**), deglycosylation of Gc protein by α-N-acetylgalactosaminidase (Nagalase) (**b**) and enzymatic preparation of GcMAF (**c**). *, inflammation-primed B cells: B cells can be treated with an inflamed membranous lipid metabolite, for example, lysophosphatidylcholine (lyso-Pc).

When peripheral blood mononuclear cells (PBMCs) containing monocytes/macrophages (macrophages for short) and lymphocytes of HIV-infected patients were treated with lyso-Pc (1 μg/ml) and cultured in a serum-free medium containing purified Gc protein (1 ng/ml) or healthy human serum (0.1%) as a source of Gc protein, the lyso-Pc-primed lymphocytes of all patients were fully capable of converting Gc protein to MAF, resulting in activation of their macrophages [Yamamoto et al., 1995]. However, cultivation of lyso-Pc-treated patient PBMCs in medium containing patient own serum (0.1%) resulted in lack or reduced levels of macrophage activation [Yamamoto et al., 1995; Yamamoto, 1997, 1999], as a consequence of lost or decreased MAF precursor activity of the patient serum Gc protein [Yamamoto et al., 1995; Yamamoto, 1997, 1999]. Loss of MAF precursor activity is due to deglycosylation of serum Gc protein by α-N-acetylgalactosaminidase (termed Nagalase) secreted from HIV-infected cells (Fig. 1b) [Yamamoto et al., 1995; Yamamoto, 1997, 1998b, 2006]. Since Nagalase is an intrinsic component of the envelope gp120 [Yamamoto, 1999, 2006], serum Nagalase activity is the sum of enzyme activities carried by both HIV virions and unassembled envelope proteins (gp160 and gp120) released from HIV-infected cells. Because of Nagalase being an HIV viral component, serum Nagalase is already complexed with patient own immunoglobulin G (anti-HIV IgG) [Yamamoto, 1999, 2006]. The IgG-bound Nagalase retains enzyme activity that can initiate fusion for HIV-infection process and also deglycosylate Gc protein [Yamamoto, 2006]. The deglycosylated Gc protein cannot be converted to MAF and as a cosequence macrophages cannot be activated, leading to the development of immunosuppression [Yamamoto et al., 1995; Yamamoto, 1999, 2006]. As disease advances serum Nagalase activity increases while the MAF precursor activity decreases [Yamamoto et al., 1995; Yamamoto, 2006]. Lack of macrophage activation and severe immunosuppression explain why AIDS patients die from overwhelming infection (e.g., pneumonia) [Gloeb et al., 1992; Stein et al., 1992; Yamamoto et al., 1995; Yamamoto, 2006].

However, exogenously given MAF can bypass the deglycosylated Gc protein and directly act on macrophages for activation. Stepwise treatment of purified Gc protein with immobilized β-galactosidase and sialidase generates probably the most potent MAF (termed GcMAF) [Yamamoto, 1993, 1996, 1998a; Yamamoto and Kumashiro, 1993; Naraparaju and Yamamoto, 1994] (Fig. 1c) ever discovered, which produces no side effects in humans [Yamamoto, 1996, 1998a; Yamamoto and Ueda, 2004a]. The optimal human dose of GcMAF, to achieve phagocytic capacity by 30-fold increased ingestion index and 15-fold increased superoxide generating capacity of peripheral blood monocytes/macrophages, was found to be ~100 ng/human

[Yamamoto et al., 2008a]. GcMAF administration greatly enhanced phagocytic and superoxide generating capacities of systemic macrophages in 3.5 hr [Yamamoto and Naraparaju, 1998]. GcMAF also has a potent mitogenic capacity to act on the progenitor cells, resulting in a 40-fold increase in systemic macrophage cell counts in 4 days [Yamamoto and Naraparaju, 1998]. Such highly activated systemic macrophages are chemotactically recruited to inflamed lesions, as manifested by a 180-fold increase in local macrophage counts [Yamamoto and Naraparaju, 1998]. The activated macrophages develop a large amount of Fc-receptors [Yamamoto and Homma, 1991; Yamamoto, 1996] as well as an enormous variation of receptors [Yamamoto et al., 2005, 2008a] that recognize a variety of antibody-bound and unbound viral and bacterial antigens and cells with surface abnormality such as cancer cells [Yamamoto et al., 2005, 2008a]. Mouse macrophages, by in vitro treatment with 10–50 pg/ml GcMAF for 3 hr, are maximally activated for phagocytosis [Yamamoto, 1996] and superoxide generation [Naraparaju and Yamamoto, 1994; Yamamoto, 1998a]. Four administrations of 100 pg GcMAF to Ehrlich ascites tumor bearing mice eradicate the tumor [Yamamoto and Naraparaju, 1997; Koga et al., 1999]. When human macrophages are treated in vitro with 100 pg/ml for 3 hr, they are highly tumoricidal and kill approximately 50% of both prostate cell line LNCaP and breast cancer cell lines MCF-7 and MDA-MB-321 in 4 hr [Yamamoto and Ueda, 2004a; Yamamoto et al., 2008a]. Weekly administrations of 100 ng GcMAF to metastatic adenocarcinoma (breast and prostate cancer) patients (n = 32) and metastatic colorectal cancer patients eradicate tumors in 16–25 weeks and 32–50 weeks, respectively [Yamamoto and Ueda, 2004a; Yamamoto et al., 2005, 2008a,b,c]. With the same therapeutic procedure (i.e., administration of 100 ng GcMAF/week) in a preliminary study of HIV-infected patients, both cell-free virions and HIV-infected cells were eradicated in 10–18 weeks [Yamamoto and Ueda, 2004b; Yamamoto et al., 2007].

MATERIALS AND METHODS

Chemicals, Reagents, and Cells

Phosphate buffered saline (PBS) contained 1 mM sodium phosphate and 0.15 M NaCl. Macrophage-SSM serum free medium was purchased from GIBCO BRL Life Science Technology (Grand Island, NY). When peripheral blood monocytes adhere to vessel substratum, they behave like macrophages which show increased synthesis of hydrolases. For manipulation in vitro and cultivation of peripheral blood mononuclear cells (PBMCs) containing monocytes/macrophages (macrophages for short) and lymphocytes (B and T cells), 0.1% egg albumin supplemented medium RPMI 1640 (EA medium) [Yamamoto, 1996] was used. Gc1F protein (the major isoform of Gc protein) was isolated from Gc1F homozygous donor serum. Lysophophatidylcholine (lyso-Pc) and p-Nitrophenyl N-acetyl-α-D-galactosaminide were purchased from Sigma Chemical Co. (St. Louis, MO). Mitomycin C, a provirus inducing agent [Sato et al., 1977], was obtained from ICN pharmaceutical, Inc. (Costa Mesa, CA). HIV-infected PBMCs were treated with 5 μg mitomycin/ml for 30 min, washed and plated on a plaque-forming indicator monolayer for detection of infectious centers [Yamamoto, 2006], or cultured in Macrophage-SSM medium supplemented with 5% FCS for 72 hr for induction of the provirus [Yamamoto, 2006]. Protein G-Sepharose to be used for precipitation of IgG-immunocoplex [Yamamoto, 2006] was obtained from Pharmacia Biotech, Inc. (Piscataway, NJ).

Procedure for Preparation of GcMAF

Procedure for preparation of GcMAF was described previously [Yamamoto, 1993; Naraparaju and Yamamoto, 1994; Yamamoto et al., 2008a,c]. Briefly, Gc1F protein was purified using 25-hydroxyvitamin D_3-affinity chromatography [Link et al., 1986]. Stepwise incubation of the purified Gc protein with immobilized β-galactosidase and sialidase yielded probably the most potent macrophage activating factor (GcMAF) ever discovered [Yamamoto and Kumashiro, 1993; Naraparaju and Yamamoto, 1994; Yamamoto, 1996] (Fig. 1c). GcMAF was filtered through a low protein-binding filter, Millex-HV (Millipore Corp., Bedford, MA) for sterilization.

The molecular structure of GcMAF is identical to that of the native human MAF. Numerous administrations (more than 10 times for 3–6 months period) of GcMAF (100–500 ng/human) to 12 humans showed no sign of side effects [Yamamoto, 1993, 1996; Yamamoto et al., 2008a,b,c]. Quality control of the preparation of GcMAF was performed for activity, sterility and safety tests.

GcMAF Therapy of HIV-Infected Patients

Participants. A cohort of 15 asymptomatic HIV-1-infected patients was included in this study. These patients must carry HIV-1 provirus in their PBMCs as demonstrated by increased Nagalase production and infectious center (plaques) formation after mitomycin treatment of PBMCs [Yamamoto, 2006]. They received GcMAF therapy exclusively and excluding combination with erythropoiesis induction. Thus, anemic HIV-infected patients were not eligible in the program. The study was approved by The Institutional Research and Ethic Committees of Nagasaki Immunotherapy Group (Nagasaki, Japan) and The Institutional Review Board of Hyogo Immunotherapy group (Hyogo, Japan). The participants gave written informed consent before entering the study.

GcMAF administration. Because the half-life of the activated macrophages is approximately 6 days [Yamamoto et al., 1988; Yamamoto et al., 2007, 2008a], 100 ng GcMAF was administered intramuscularly once a week.

Procedures Used for Prognostic Analysis

Serum and blood samples were weekly or biweekly collected immediately prior to each GcMAF administration and used for prognostic analyses. Precursor activity of serum Gc protein, serum Nagalase activity, p24 antigen, HIV-1 RNA copies, CD4 + and CD8 + T-cells counts, viral load in plasma and proviral load in PBMCs were always determined during GcMAF therapy.

The MAF precursor activity of serum Gc protein and serum Nagalase activity were determined to show recovery rate of immuno-potency during the early stage of GcMAF therapy. These prognostic analyses were compared with amounts of p24 antigen and HIV-1 RNA and changes in CD4 + and CD8 + T-cell counts. Since serum Nagalase activity is the total sum of Nagalase activities carried by HIV virions and unassembled envelope proteins in the patient blood stream and is detectable until complete eradication of HIV infection [Yamamoto, 1999, 2006], assessment of curative response to GcMAF therapy was performed by determining serum Nagalase activity as a prognostic index during the entire kinetic course of GcMAF therapy.

CD4 and CD8 Cell Counts

The blood samples were processed immediately within 2 hr of collection, for determining the absolute counts of CD4 + cells and CD8 + cells by two color immunophenotyping on the single platform fluorescence activated cell sorting (FACS) count system (Becton Dickinson Pvt. Ltd, Mountain View, CA), using fluorochrome labeled monoclonal antibodies to CD4 + and CD8 + T-cells, following manufacurer's instructions.

Immunoassay of HIV-1 p24 Antigen

HIV p24 ELISA (Lentivirus Quantitation Kit, Cell Biolabs, Inc., San Diego, CA) was used.

An anti-HIV p24 monoclonal coating antibody is adsorbed onto a microtiter plate. P24 antigen present in the sample or standard binds to the antibodies adsorbed on the plate, a biotin-conjugated goat anti-p24 antibody is added and binds to p24 antigen captured by the first antibody. Following incubation and wash steps, Streptavidin-HRP is added and bind to the biotin conjugated anti-p24. Following unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of p24 antigen present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from recombinant HIV-p24 protein and sample p24 concentration is then determined.

Detection of HIV-1 RNA in Serum by RT-PCR

HIV-1 RNA in serum was measured by a RT-PCR kit (Amplicator HIV-1 Monitor, Roche Diagnostic Systems, Basel, Switzerland) as described by Hashida et al. [2000]. The cutoff value of this assay is 400 copies/ml.

Determination of MAF Precursor Activity of Serum Gc Protein of HIV-Infected Patients

Assay procedure for determination of MAF precursor activity of patient serum Gc protein has been described previously [Yamamoto et al., 1995, 1996, 1997; Koga et al., 1999; Yamamoto, 2006]. Briefly, healthy control PBMCs were treated with lyso-Pc (1 μg/ml) for 30 min and cultured in EA medium containing 0.1% serum of HIV-infected patients or healthy controls as a source of Gc protein. After 3 hr cultivation the extent of macrophage activation was assayed for superoxide generating capacity [Yamamoto et al., 1996; Yamamoto, 1997]. The data were expressed as nmol of superoxide produced per minute per 10^6 cells (macrophages). These values represent the MAF precursor activity of patient serum Gc protein [Yamamoto et al., 1996; Yamamoto, 1997]. Lost or reduced MAF precursor activity of patient serum Gc protein are exhibited as a decrease in superoxide generation as compared with control healthy human Gc protein. Thus, this procedure measures the ability of individual patient to activate macrophages as for immune potential.

Cultivation of the mixture of lyso-Pc-treated lymphocytes and macrophages in EA medium without addition of serum results in the production of 0.5–0.8 nmol superoxide/min/10^6 macrophages [Yamamoto et al., 1994]. Thus, if patient serum (0.1%) generates < 0.8 nmol superoxide/min/10^6 macrophages, the precursor activity of the patient serum Gc protein is considered to be lost.

Detection of Nagalase in HIV-Infected Patient Sera

Patient sera (300 μl) were precipitated with 70% saturated ammonium sulfate. The precipitates were dissolved in 50 mM sodium citrate buffer (pH 6.0) and dialyzed against the same buffer at 4°C for 2 hr. The dialysates were made up to 1 ml in volume and assayed for Nagalase activity [Yamamoto et al., 1995, 1996, 1997, 2008a]. Substrate solution (250 μl) contained 5 μmol of p-nitrophenyl N-acetyl-α-D-galactosaminide in 50 mM citrate buffer (pH 6.0). The reaction was initiated by addition of 250 μl of the dialyzed samples, kept at 37°C for 60 min and terminated by adding 200 μl of 10% TCA. After centrifugation of the reaction mixture, 300 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 and expressed as nmol/min/mg protein [Yamamoto et al., 1995, 1996, 1997, 2008a]. Protein concentrations were estimated by the Bradford [1976] method.

Healthy control sera exhibit very low levels (0.35–0.68 nmol/min/mg) of the enzyme activity. This is the enzyme activity of α-galactosidase that can also catabolize the chromogenic substrate (i.e., p-nitrophenyl N-acetyl-α-D-galactosaminide) for Nagalase [Yamamoto

et al., 1995, 1996, 1997]. A reduction in serum Nagalase activity to 0.68 nmol/min/mg or less in patient during GcMAF therapy serves as demonstration that HIV-infection has been eradicated.

Determination of the Viral Load in Plasma and the Proviral Load in PBMCs of the HIV-Infected Patients

Assay methods for plaques for viral load in plasma and plaques as infectious centers for proviral load in PBMCs were employed. Monolayers of MT-4 cells were prepared for plaque assay [Harada et al., 1986; McKeating et al., 1989]. Six well tissue culture plates were treated with 50 μg/ml of poly-L-lysine for 1 hr at room temperature. The wells were then washed three times with distilled water and left to dry in air. MT-4 cells were washed in serum free EA medium twice and placed at a final cell concentration of 2×10^6/well. Cells were then allowed to adsorb to the plates for 30 min at room temperature. Unbound cells were subsequently removed by aspiration.

For determination of the viral load in patient plasma as the amount of cell-free virions, patient plasma (at 100 μl) were added to the cells. After adsorption of virus for 1 hr at room temperature, the cells were overlaid with 1.5 ml of 0.6% molten agarose (Sea Plaque agarose, FMC Bioproducts, Rockland, ME) containing nutrient medium (RPMI-1640 medium supplemented with 10% FCS, 100 IU of penicillin and 100 μg of streptomycin per ml). After the agar overlay solidified, the plates were incubated in a CO_2 incubator at 37°C. After 3 days of incubation, the cells were overlaid with another 1.5 ml of agarose medium. After 5 or 6 days of incubation, some plaques were visible.

The HIV virions in plasma of some patients do not produce distinct plaques. The plaques were then fixed and stained with peroxidase-labeled antibodies [Matsui et al., 1987]. Three milliliters of 95% methanol was poured onto each dish, and the cells were fixed overnight at room temperature. Methanol was changed three to four times during fixation. After fixation, 3 ml of formamide was added to each dish to melt the agarose. The dishes were washed with running water and 1 ml of 1:1,000-diluted human antiserum against HIV was added to each dish. After incubation for 1 hr at 37°C, the dishes were washed three times with PBS, and 1 ml of biotinylated anti-human immunoglobulin G (1:500 dilution in PBS; Amersham International plc, Buckinghamshire, England) was added and incubated for 1 hr at 37°C. The dishes were washed three times with PBS, and 1 ml of streptavidin-biotinylated horseradish peroxidase complex (1:1,000 dilution in PBS; Amersham) was added and incubated for 1 hr at 37°C. After dishes were washed with PBS three times, brownish stained spots were visualized by the addition of 1 ml of a staining mixture containing 0.5 mg of 3,3'-diaminobenzidine tetrahydrochloride per ml and 0.02% H_2O_2 in PBS. When the stain had reached a suitable intensity, the dishes were washed with running water and dried. The numbers of the immunostained plaques were counted.

For determination of the numbers of HIV-infected PBMCs as the proviral load in the PBMCs, 0.1 ml of mitomycin-treated patient's PBMCs was added to1.5 ml of 0.6% molten agarose (Sea Plaque agarose, FMC Bioproducts, Rockland, ME) containing nutrient medium (RPMI-1640 medium supplemented with 10% FCS, 100 IU of penicillin and 100 μg of streptomycin per ml). After the agar overlay solidified, the plates were incubated in a CO_2 incubator at 37°C. After 3 days of incubation, the cells were overlaid with another 1.5 ml of agarose medium. Neutral red stain was added in the second agarose overlay at a concentration of 0.00032%, incubated in the dark for 20 hr and the plaques for cell-free virions and plaques for infectious centers were counted using an inverted microscope [Yamamoto, 2006]. Since an infectious center is produced by infection of MT-4 cells with a large number of HIV virions released from mitomycin-treated cells, peroxidase-labeled immunostaining is generally not required for detection of infectious centers.

The total assessment of viral load in the HIV-infected patients is accomplished by the plaques for cell-free HIV virions and the infectious centers for determination of HIV-infected patient PBMCs. Since the macrophages activated by GcMAF phagocytize cell-free HIV virions, eradication of the proviral carrying PBMCs follows that of cell-free virions. If no infectious centers are detected after GcMAF therapy, HIV-infection is eradicated.

RESULTS

MAF Precursor Activity of Serum Gc Protein and Serum Nagalase Activity of HIV-Infected Patients

Because the fate and staging of the HIV-infected patients correlate with the level of immunosuppression and viral load [Yamamoto et al., 1995; Yamamoto, 1999, 2006], the MAF precursor activity of Gc protein and serum Nagalase activity for each of 15 patients were determined before entering GcMAF therapy. As shown in Table I, patients having lower MAF precursor activity had higher serum Nagalase activity. This inverse correlation between the MAF precursor activity and serum Nagalase activity is consistently observed with a number (>230) of HIV-infected patients screened [Yamamoto et al., 1995; Yamamoto, 1997, 1999, 2006]. In contrast, quantitative correlation between the MAF precursor activity (or serum Nagalase activity) and CD4+value of the patients has not been feasible [Yamamoto et al., 1995; Yamamoto, 1997, 1999]. The majority of this HIV patient group carries low CD4+cell counts ranging from 156 to 336 cells/μl with a few exception (Table I). However, only a small proportion of cells expressing CD4+cells is infected [Schnittman et al., 1989; Gougeon et al., 1993]. Thus, CD4+cell counts do not reflect the frequency of HIV-infection [Yamamoto et al., 1995] and could not be used for quantitative kinetic study of GcMAF therapy.

TABLE I. Precursor Activity of Serum Gc Protein and Nagalase Activity Detected in the Blood Stream and CD4 + Cell Counts of 15 HIV-Infected Patients

Patient No.	Precursor activity[a] superoxide produced (nmol/min/10^6 cells)	Nagalase specific activity (nmol/min/mg)	Pre-GcMAF therapy CD4 + cells/μl	Post-GcMAF therapy CD4 + cells/μl
1	0.85	5.38	236	828
2	1.53	4.59	326	725
3	1.94	3.72	312	626
4	1.72	4.08	475	818
5	1.10	5.02	156	784
6	0.88	5.42	224	997
7	1.62	4.56	282	807
8	2.73	4.19	422	652
9	2.80	4.98	253	908
10	2.05	3.77	326	561
11	2.58	3.18	336	846
12	0.72	5.58	214	794
13	2.76	3.06	526	883
14	2.78	4.04	312	787
15	1.63	4.88	264	942
Control[b]	6.25	0.23[c]	824	

[a]Precursor activity was measured by superoxide generating capacity of healthy human macrophages when treated with 0.1% of patient's serum as a source of Gc protein.
[b]Healthy control (average of 5).
[c]This uninfected human enzyme is known to be α-galactosidase [Yamamoto et al., 1995, 1996]. This enzyme is unable to deglycosylate Gc protein though it is able to hydrolyze *p*-nitrophenyl *N*-acetyl-α-D galactosaminide [Yamamoto et al., 1995, 1996]. Because α-*N*-acetylgalactosaminidase and α-galactosidase are evolutionary conserved, carry 46.9% amino acid sequence homology and share a common chromogenic substrate for their catabolic capacities [Yamamoto et al., 1995, 1996, 1997].

Demonstration of an Excess Antibodies Beyond Anti-HIV Antibodies Already Complexed With Serum Nagalase in HIV-Infected Patient Blood Stream

Because of Nagalase being a viral component, Nagalase in the patient blood stream was complexed with patient's own immunoglobulin G (i.e., polyclonal anti-HIV IgG) [Yamamoto, 1999, 2006]. To test whether each patient has an excess amount of anti-HIV IgG antibodies beyond antibodies already complexed with serum Nagalase, 1 ml of a patient serum was mixed with a culture lysate (1 ml) of another patient HIV-infected PBMCs which have been treated with Mitomycin C (MC: a provirus inducer [Sato et al., 1977]) and cultured for 37°C for 72 hr [Yamamoto, 2006]. After 1 hr incubation of the mixture at 37°C, protein G-Sepharose (40 mg) was added and centrifuged to isolate IgG-Nagalase immunocomplexes. The supernatant was assayed for Nagalase activity. Precipitated protein G-Sepharose bound IgG-Nagalase immunocomplexes were washed with 50 mM citrate phosphate buffer (pH 6.3), and the enzyme was eluted from the protein G-Sepharose with citrate phosphate buffered saline containing 0.15 M NaCl (pH 6.5) and assayed for Nagalase activity [Yamamoto, 2006]. As shown in Table II, the total amount of Nagalase activity was precipitated with protein G-Sepharose. The result indicates that sera of all four HIV-infected patients had ample amounts of anti-HIV IgG antibodies to form immunocomplexes with the exogenously given Nagalase which has been mitomycin-induced from HIV-infected PBMCs of another patient. This reveals that HIV-infected patients have an ample amount of anti-HIV IgG antibodies capable of complexing with Nagalase as soon as secreted from HIV-infected cells.

MAF Precursor Activity of Gc Protein and Serum Nagalase Activity as Prognostic Parameters During GcMAF Therapy of HIV-Infected Patients

MAF precursor activity and serum nagalase activity during GcMAF therapy. When systemic macrophages are activated by administration of 100 ng GcMAF, macrophages develop a large amount of Fc-receptors as well as an enormous variation of receptors [Yamamoto and Homma, 1991; Yamamoto, 2002; Yamamoto et al., 2008a,b,c]. Since serum Nagalase is immunocomplexed with the patient's own immunoglobulin G (anti-HIV IgG), we anticipated that the macrophages activated by GcMAF rapidly phagocytize IgG-bound Nagalase via Fc-receptor mediation. Thus, the two prognostic indices, MAF precursor activity of serum Gc protein and serum Nagalase activity of the first five patients (Table I) were analyzed during the first 6 weeks of GcMAF therapy.

Because the half-life of activated macrophages is approximately 6 days [Yamamoto et al., 1988; Yamamoto, 1996; Yamamoto et al., 2007, 2008a], 100 ng of GcMAF were administered weekly and patient serum samples were collected weekly at the end of a week but immediately prior to each GcMAF administration. As GcMAF therapy progressed, the MAF precursor activity of all five patients increased while their Nagalase activity decreased inversely as shown in Table III. Because serum Nagalase activity is the sum of

TABLE II. HIV-Infected Patients Carry Ample Amounts of Anti-HIV-1 IgG Capable of Complexing With Nagalase Activity Secreted From HIV-Infected Patient PBMCs

Patient No.	Serum	MC-induced HIV-PBMCs[a]	Nagalase (nmol/min/mg) Control[b]	Protein-G: precipitate[c]	Supernatant[d]
S1	+	−	5.38	5.06	0.22
	−	+	3.18		
	+	+	8.52	8.22	0.43
S2	+	−	4.59	4.35	0.38
	−	+	2.29		
	+	+	6.78	6.62	0.36
S3	+	−	3.72	3.16	0.33
	−	+	2.86		
	+	+	6.12	6.08	0.59
S4	+	−	5.42	5.26	0.32
	−	+	3.56		
	+	+	8.84	8.59	0.73

[a]Culture medium of Mitomycin C-induced HIV-infected PBMCs of another patient.
[b]Sixfold diluted patient serum (300 μl), Mitomycin-induced PBMCs culture medium and their mixture were assayed for Nagalase activity [Yamamoto, 2006].
[c]Nagalase-antibody complex was eluted from protein G-Sepharose with 0.1 M citrate and assayed for Nagalase activity. [d]Supernatant after protein G-Sepharose precipitation was assayed for the enzyme.

Nagalase activities carried by HIV virions and unassembled envelope proteins, as serum Nagalase activity decreased the amount of HIV virions in the blood stream decreased while the MAF precursor activity as the immuno-potency increased.

Effect of GcMAF therapy on quantity of HIV-1 RNA and p24 antigens and cell counts of CD4 + and CD8 + T-cells. Nagalase is the major viral constituent required for infectious process [Yamamoto, 2006]. Loss or decrease of serum Nagalase activity during GcMAF therapy was always compared with quantitative change of HIV-1 RNA and p24 antigen and cell counts of CD4 + and CD8 + T-cells. Table III shows that as GcMAF therapy progressed both RNA copy number and p24 antigen rapidly decreased and often reached to undetectable levels at the 6th week of GcMAF therapy. Furthermore, as Nagalase activity decreased during GcMAF therapy, CD4 + cell counts increased while CD8 + cell counts decreased.

Eradication of nagalase carriers. Since patient anti-HIV antibodies do not inhibit Nagalase activity [Yamamoto, 2006], the antibodies of IgG-Nagalase immunocomplexes are bound to the structure of Nagalase carriers (i.e., HIV virions and envelope proteins) other than the active center of Nagalase. As shown in Figure 2a, weekly decrease of serum Nagalase activities of five patients suggests that IgG-bound Nagalase carriers in the patient blood stream were rapidly phagocytized by the activated macrophages via Fc-receptor meidation. Although serum Nagalase carriers were replenished by spontaneous induction from HIV-infected cells, serum Nagalase activity decreased steeply during the first 6 weeks of GcMAF therapy (Table III and Fig. 2a). We continued to assay serum Nagalase activity as a measure for a decrease in the amount of Nagalase carriers. Since spontaneous induction for HIV-infected cells to release HIV virions is at a high frequency [Yamamoto, 2006], continuous removal of Nagalase carriers by the activated macrophages may soon result in exhaustion of HIV-infected cells. Time course weekly prognostic analysis of GcMAF therapy was continued for additional 6–12 weeks. As shown in Figure 2a, the Nagalase activities of patients #2, 3, 4, and 5 reached to the control level in 10–13 weeks, indicating eradication of both Nagalase carriers and HIV-infected cells while the Nagalase activity of patient #1 reached to the control level in 18 weeks. When these patient PBMCs were treated with mitomycin C and plated on MT-4 cell monolayers, no infectious centers were detectable (Table IV), indicating eradication of HIV-infection. From these results we conclude that GcMAF therapy of HIV patients cures HIV disease.

Time Course Study of Serum Nagalase Activity of HIV-Infected Patients During GcMAF Therapy

Based on findings of the curative process of HIV-infection with GcMAF therapy of five HIV-infected patients described in the preceding section, we conclude that eradication of both cell-free HIV virions and HIV-infected cells by GcMAF therapy can be monitored by serum Nagalase activity as a prognostic index and confirmed by no infectious center formation of mitomycin-treated PBMCs. This conclusion deserves substantiation with GcMAF therapy of more HIV-infected patients. Time course analyses of serum Nagalase activity of additional ten HIV-infected patients were performed to assess the efficacy of GcMAF therapy for HIV disease. Serum Nagalase activities of these patients were analyzed weekly for the first 2 weeks and followed by biweekly analysis up to 18 weeks. These patients had the initial Nagalase activities ranging from 3.06 to 5.58 nmol/min/mg (Table I). As shown in

TABLE III. Correlation Between Serum Nagalase Activity, Quantity of p24 Antigen and HIV-1 RNA and Cell Counts of CD4+ and CD8 + T-Cells of Five HIV-1 Infected Patients During GcMAF Therapy

Patient No.	Assayed weeks	Precursor activity (nmol)	Nagalase (nmol/min/mg)	p24 (pg/ml)	RNA (copies/ml)	CD4 (cells/μl)	CD8 (cells/μl)
1	0	0.85	5.39	343	432,000	236	1,174
	1	1.52	4.65				
	2	1.94	3.72	85	58,000	495	852
	3	2.32	3.23				
	4	2.61	2.75	22	7,200	648	598
	5	2.72	2.40				
	6	2.88	2.22	4.2	900	806	583
2	0	1.53	4.59	127	386,000	326	1,023
	1	1.85	3.77				
	2	2.10	3.18	34	48,000	504	689
	3	2.66	3.62				
	4	3.13	1.82	7.2	5,200	692	593
	5	3.54	1.05				
	6	3.96	0.78	<0.2	UD	716	599
3	0	1.94	3.72	68	165,000	312	897
	1	2.46	3.04				
	2	2.82	2.21	17	11,600	513	605
	3	3.65	1.60				
	4	3.72	1.16	<0.2	1,400	615	525
	5	3.86	1.04				
	6	4.04	0.80	<0.2	UD	632	593
4	0	1.72	4.08	92	238,000	475	1,010
	1	1.98	3.38				
	2	2.59	2.72	19	28,000	608	754
	3	2.96	2.08				
	4	3.22	1.63	4.8	2,300	802	585
	5	3.36	1.36				
	6	3.48	1.14	<0.2	UD	811	536
5	0	1.10	5.02	305	378,000	156	1,085
	1	1.72	4.24				
	2	1.98	3.54	69	52,000	525	834
	3	2.54	2.98				
	4	2.70	2.42	13	3,800	705	712
	5	2.96	2.11				
	6	3.26	1.78	<0.2	UD	788	558
Control (average of 5)		4.25	0.45[a]			872	524

UD indicates undetectable copies less than the cutoff value of 400 copies/ml.
[a]Healthy subjects carry α-galactosidase that shares the same substrate with Nagalase [Yamamoto et al., 1995].

Figure 2b, the serum Nagalase activity of all 10 patients decreased as GcMAF therapy progressed. Thus, the activated macrophages rapidly phagocytized IgG-bound Nagalase carriers. After less than 18 weekly administrations of GcMAF, their serum Nagalase activities decreased to the healthy control value (<0.60 nmol/min/mg), suggesting that these patients were free of both HIV virions and HIV-infected cells. In fact, these patients plasma did not produce plaques (Table IV). When these patient PBMCs were treated with mitomycin C and plated on MT-4 cell monolayers [Yamamoto, 2006], no infectious centers were detectable, indicating eradication of HIV-infection as shown in Table IV. All 15 patients carried healthy CD4 + cell counts, 798 ± 213 cells/μl (Table I). During 7 years observation after completion of GcMAF therapy, these patients showed no increase in their serum Nagalase activities, indicating no recurrence of the disease. HIV-1 RNA and p24 antigen were never detectable in these patient blood stream. Furthermore, the above healthy CD4 + cell counts of these patients were maintained for the entire 7-year period after GcMAF therapy.

DISCUSSION

HIV-1 infection appears to induce some antibodies in patients who nonetheless develop inadequate immunity for the disease [Yamamoto, 2006]. Consequently, a persistent infection is established. Although HIV-1 is one of the retroviruses, it has lytic capacity upon infection to susceptible host cells. This characteristic of HIV-1 allows plaque assay for quantitation of the virions. If cells did not lyse by HIV-1 infection, however, the cells become latently infected but the HIV provirus is rather unstable [Yamamoto, 2006]. Thus, the cells latently infected with HIV constantly release HIV virions and unassembled envelope proteins carrying Nagalase activity into the patient blood stream

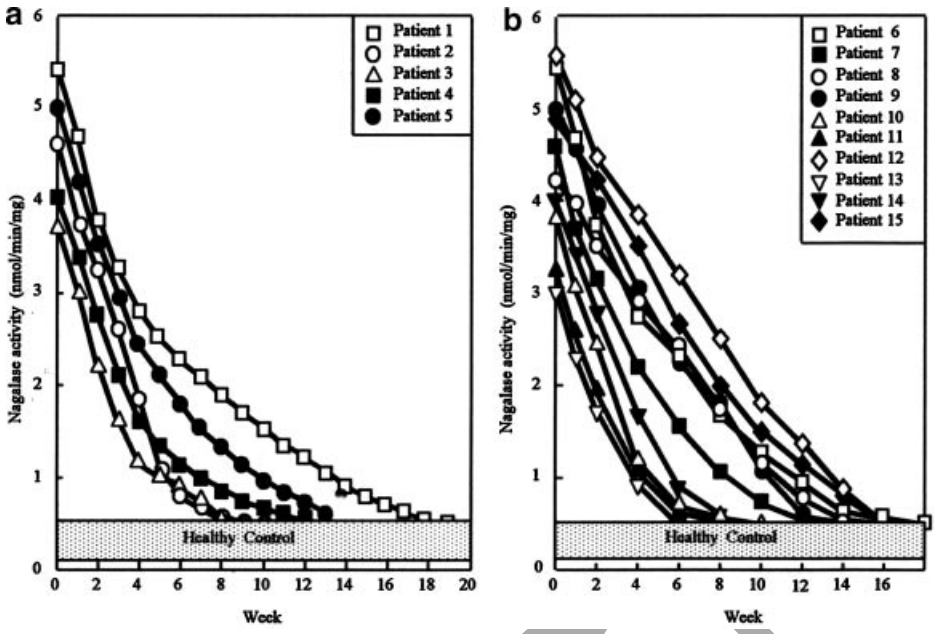

Fig. 2. Therapy of HIV-infected patients with GcMAF. Time course analysis of serum Nagalase activity as a prognostic index during GcMAF therapy of five HIV-infected patients (**a**) and additional ten patients (**b**).

[Yamamoto, 2006]. The latency of HIV-1 provirus was also confirmed by increased Nagalase production and infectious center (plaque) formation after mitomycin treatment of patient PBMCs [Yamamoto, 2006]. Because patient sera contain ample amounts of anti-HIV IgG, these spontaneously released Nagalase carriers were immediately immunocomplexed with anti-HIV antibodies. These antibodies are largely not neutralizing antibodies because of blood borne infectivity of HIV, although Wendler et al. [1987] reported that some patients develop neutralizing antibodies. The IgG-bound virions still retain Nagalase activity [Yamamoto, 2006] that is required for infectivity of the HIV virions and also able to deglycosylate serum Gc protein [Yamamoto, 2006]. The deglycosylated Gc protein cannot be converted to MAF, leading to the development of immunosuppression [Yamamoto et al., 1995; Yamamoto, 1997, 1999].

Monocytes/macrophages activated by GcMAF administration immediately stop DNA replication and rapidly synthesize a large amount of Fc-receptors as well as an enormous variation of receptors [Yamamoto and Homma, 1991; Yamamoto, 2002; Yamamoto et al., 2005, 2008a]. The macrophages activated by GcMAF phagocytize multi-IgG-complexed larger object (i.e., virion) more quickly than IgG-complexed protein (i.e., envelope protein). This may explain why HIV-1 RNA copies decreased rapidly during GcMAF therapy as shown in Table III. Although these activated macrophages constantly intercept both IgG-bound and unbound HIV virions as soon as secreted from HIV-infected cells to prevent reinfection, they preferentially phagocytize IgG-bound virions rapidly via Fc-receptor mediation [Yamamoto and Homma, 1991; Yamamoto, 2002]. Since spontaneous induction of HIV-infected cells to release HIV virions is at a high frequency [Yamamoto, 2006], continuous removal of cell-free HIV virions by the activated macrophages soon results in exhaustion of HIV-infected cells. This explains why HIV-infected patients are rapidly cured by GcMAF therapy. Eradication of HIV-infection was firmly confirmed by no infectious centers produced by mitomycin-treated patient PBMCs after GcMAF therapy (Table IV).

Envelope hemagglutinating protein HA-1 of influenza virus also carries Nagalase activity which is detectable at a level of 1.5–2.0 nmol/min/mg in patient sera immediately after influenza infection [Yamamoto and Urade, 2005]. Upon infection of HIV the hosts develop flu-like symptoms with serum Nagalase activity similar to the influenza acute state. Like serum Nagalase

TABLE IV. Viral Load in Plasma and Proviral Load in PBMCs During GcMAF Therapy of 15 Patients

	Before therapy	4th week	Post-therapy[a]
Median plasma viral load (plaques/ml)	6.7×10^2	1.6×10^1	0
Median proviral load (infectious centers/ml)	1.1×10^3	1.2×10^2	0

[a]Patient serum Nagalase activities reached to the healthy control level by 10–18 weeks.

activity of influenza virus-infected patients, serum Nagalase activity is the sum of enzyme activities carried by HIV virions and unassembled envelope proteins released from HIV-infected cells. Thus, serum Nagalase activity is readily detectable at all stages of HIV-infection [Yamamoto et al., 1995; Yamamoto, 2006] whereas HIV-1 RNA and p24 protein are undetectable in the early stage of HIV-infection [Hashida et al., 2000]. Similarly after 6 weeks of GcMAF therapy the majority of patients, HIV-1 RNA and p24 antigen are undetectable (Table III). Because of the availability of the precision measurement of serum Nagalase, the cure rate measurements of HIV-infection during GcMAF therapy have been possible. Therefore, the significance of GcMAF therapy of HIV-infection has been greatly enhanced by the discovery of HIV-specific Nagalase that can accurately monitor the cure rate during GcMAF therapy of HIV-infected patients.

In the present study GcMAF therapy was given to nonanemic HIV-infected patients and found to be highly curative. Anemia is a common manifestation of HIV infection, occurring in approximately 30% of patients with asymptomatic infection and in as many as 75–80% of patients with AIDS [Levine et al., 2001]. A number of studies have consistently found anemia to be associated with reduced survival [Moore, 1999]. Anemic HIV and AIDS patients have yet to be tested with GcMAF therapy.

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