Potentiation of T Cell Function by a Marine Algae-Derived Sulfated Polymannuroguluronate: In Vitro Analysis of Novel Mechanisms

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Abstract. Marine algae-derived sulfated polymannuroguluronate (SPMG), a candidate drug for AIDS treatment, was intraperitoneally injected into normal mice for 6 weeks, and the in vivo and in vitro mechanisms of SPMG for immunomodulation were investigated in isolated lymphocytes by MTT assay, flow cytometry, and surface plasmon resonance assay. SPMG treatment at 5 and 10 mg/kg enhanced concanavalin A (ConA)-induced T cell proliferation, cellular levels of CD69, interleukin-2 (IL-2), and interferon-γ (IFN-γ), as well as CD4/CD8 ratio, while decreasing tumor necrosis factor-α (TNF-α) level in T cells of peripheral blood mononuclear cells. In addition, 1 molecule of SPMG bound to 2/3 molecules of IL-2 with a K_D of 9.53 × 10^{-7} M. Heparin prevented SPMG binding to IL-2 by 72.2%; thus, to a large extent, SPMG and heparin share common binding sites on IL-2. In contrast, other glycosaminoglycans (e.g., chondroitin sulfate and dermatan sulfate) had little effect on SPMG and IL-2 interaction, suggesting the requirement of a defined sequence within the sugar chain for specific recognition of IL-2. Concomitant treatment of IL-2 and SPMG augmented lymphocyte proliferation, compared with IL-2 alone; in contrast, SPMG alone had no proliferative effect. Taken together, our findings demonstrated for the first time that SPMG exerted its immunomodulation by direct activation of T cell function, accompanied by simultaneous modulation of cytokine function, which suggests that SPMG would show great promise for use in anti-AIDS therapy.

Keywords: sulfated polymannuroguluronate, immunomodulation, T lymphocytes, cytokine

Introduction

The primary immunological abnormality resulting from human immunodeficiency virus (HIV) infection is progressive depletion and functional impairment of T cells expressing CD4 antigen (1). The ability of HIV-1 to selectively infect, replicate in, and destroy CD4+ T cells accounts, to a certain degree, for the loss of CD4+ cells and consequent systemic immunodeficiency (2).

Current views hold that the most promising pharmacological activities of polysaccharides are characterized by their immunopotentiating functions, such as activation of immunocytes and modulation of immune functions (3–5). Sulfated polymannuroguluronate (SPMG), a kind of sulfated polysaccharide extracted from brown algae Laminaria japonica with specific means of fractionation and chemical modification, bears rich 1,4-linked β-D-mannurionate blocks with modified sulfate group, with the average molecular weight of 8000 Da. Our previous in vitro and in vivo studies have demonstrated a significant inhibition by SPMG of the replication of HIV and simian immunodeficiency virus (SIV) (6). SPMG has been approved as an anti-acquired immune deficiency syndrome (AIDS) candidate, now entering a Phase II clinical trial in China. The mechanisms of action underlying the anti-HIV-1 activity of SPMG has been elucidated to be attributable to its inhibitory effect on the process of HIV entry mainly via its interaction with the V3 domain of gp120, which subsequently blocked V3 binding to CXCR4 or CCR5 coreceptors on the host cell surface (7). SPMG also augmented dramatically humoral immune response, as shown by a significant increment in antibody titres in

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SIIVmac239-infected Chinese macaques and afforded its immunomodulating activities in both normal and immuno-suppressive mice (8). Much remains, however, unknown about the immunomodulation mechanism of SPMG. In this paper, we firstly focused on the in vivo effects of SPMG on T lymphocyte proliferation in normal mice and analyzed the SPMG mechanisms by several in vitro systems.

Materials and Methods

Drugs and reagents

Sulfated polymannoguluronate (SPMG) was provided by Professor Guan Huashi, Marine Drug and Food Institute, Ocean University of China, P.R. China (Lot number: 20001101). Phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A (BFA), ConA, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sulfo-NHS-biotin, and streptavidin were purchased from Sigma, St. Louis, MO, USA. Complete RPMI 1640 medium was obtained from Gibco, Grand Island, NY, USA. The following monoclonal antibodies (mAbs) were purchased from Becton Dickinson, San Diego, CA, USA: rat-anti-mouse-CD8-FITC; rat-anti-mouse-CD3-PE; rat-anti-mouse-CD4-PE; rat-anti-mouse-CD3-Cy; rat-anti-mouse-CD3-FITC; hamster-anti-mouse-CD69-PE; rat-anti-mouse-IL-2-PE; rat-anti-mouse-IFN-γ-PE; rat-anti-mouse-TNF-α-PE; rat IgG2b, κ-Cy; rat IgG2a, κ-FITC; rat IgG2a, κ-PE; and hamster IgG-PE. The CM5 biosensor chip was purchased from Amersham Pharmacia, Biacore AB, Uppsala, Sweden.

Animals

Male Balb/c mice (body weight 18 – 20 g; Chinese Academy of Medical Sciences) were housed in groups in a controlled temperature (20 ± 2°C), with free access to standard food and tap water. Fifty mice were randomly divided into five groups; they were intraperitoneally injected once daily for consecutive 6 weeks with either saline (control group) or SPMG at 2.5, 5, 10, or 20 mg/kg, respectively.

Lymphocyte proliferation assay

Mice were sacrificed by cervical dislocation. Thymocytes were dissociated through a stainless steel mesh, suspended in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin; and plated in 96-well round bottom plates in sextuple for proliferation, giving the final cell density of 6 × 10⁵ per ml. Cells were treated without or with 5 μg/ml ConA for 48 h at 37°C in 5% CO₂/100% humidified air, and the cell proliferation was measured by MTT assay; data obtained were calculated according to the following formulation:

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\text{Proliferative index} = \frac{\text{A value of ConA-stimulated cells}}{\text{A value of nonstimulated cells}}
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To measure the acute in vitro effect of SPMG on lymphocyte proliferation, SPMG was mixed without or with IL-2 at room temperature (RT) for 10 min prior to the experiments; then, cells were treated without or with 50 IU/ml IL-2 in the absence or presence of SPMG at 1, 10, and 100 μg/ml for 48 h and subjected to MTT assay.

Cell surface expression of CD69

Thymocytes (1 × 10⁶ per well) were treated without or with 2.5 μg/ml ConA for 6 h, and harvested in 100 μl of washing solution (phosphate-buffered saline (PBS) plus 0.5% bovine serum albumin and 0.1% NaN₃). Then, cells were stained with anti-CD69-PE for 30 min at RT, while conjugated mAb served as the isotype control (hamster IgG-PE). After they were washed twice, cells were fixed by 200 μl of a 1% (W/V) solution of paraformaldehyde in PBS and stored at 4°C until analyzed within 24 h by flow cytometry (9).

Evaluation of peripheral blood CD4 and CD8 lymphocyte subsets

Heparinized peripheral blood was treated with a mixture of anti-CD3-Cy, anti-CD4-PE, and anti-CD8-FITC mAbs; conjugated mAbs were used as the isotype control (rat IgG2b, κ-Cy; rat IgG2a, κ-PE; rat IgG2a, κ-FITC). After 30-min incubation at RT, erythrocytes were lysed in 2 ml of 1 × lysing solution for 10 min at RT. After they were washed twice, cells were fixed by 200 μl of a 1% (W/V) solution of paraformaldehyde in PBS and stored at 4°C until analyzed within 24 h by flow cytometry (10).

Detection of intracellular IL-2, IFN-γ, and TNF-α

Heparinized peripheral blood was diluted (1:1) with RPMI 1640 medium in polypropylene conical tubes and activated by 50 ng/ml PMA plus 1 μg/ml ionomycin for 6 h at 37°C in 5% CO₂/100% humidified air; at 4 h prior to the end of activation, 10 μg/ml BFA was added. Incubation was terminated by adding 10 μg/ml of cold 20 mM EDTA; cells were subjected to vortexing followed by another 15 min-incubation at RT. Cells were centrifuged for 8 min at 450 × g at 4°C. Then they were permeabilized in 0.5 ml of 1 × permeabilizing solution (Becton Dickinson) for 10 min at RT, washed by centrifugation, and stained with anti-CD3-FITC and anti-IL-2-PE/anti-IFN-γ-PE/anti-TNF-α-PE mAbs, conjugated mAbs being used as the isotype control (rat IgG2b, κ-FITC; rat IgG2a, κ-PE). After they were washed


twice, cells were fixed by 200 μl of a 1% (W/V) solution of paraformaldehyde in PBS and stored at 4°C until analyzed within 24 h by flow cytometry (11 – 13).

Flow cytometry
The flow cytometer (Vantage, Becton Dickinson) was equipped with separated 488 nm excitation lines supplied by air-cooled Ar lasers. Lymphocyte gating was performed based on the FSC/SSC, CD3-Cy/SSC, or CD3-FITC/SSC light scattering. Data were obtained in a mode of 10000 gated events, and analyzed with the CellQuest software (Becton Dickinson), represented in a dot plot, and expressed as percentage of gated cells.

Surface plasmon resonance assay (optical biosensor interaction analysis evaluation)
The surface plasmon resonance assay (SPR) biosensor instrument (BIACore® X, Amersham Pharmacia) was utilized, which enables analyses of the kinetics and affinity of the interaction between molecules in a real time resolution. A polysaccharide ligand, SPMG, was biotinylated and then immobilized onto the surface of a carboxymethylated dextran sensor chip (CM5) via streptavidin-biotin coupling (14), according to the BIA applications Handbook. Briefly, the biotinylated SPMG was immobilized onto one of the flow cells (FC1) of the CM5 sensor chip surface at 25°C, with a constant flow rate (5 μl/min) of HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (V/V) polysorbate 20). Assay was initiated by injecting 80 μl of test sample dissolved in HBS-EP buffer over the SPMG-immobilized chip surface, followed by 5-min washing with HBS-EP buffer. The sensor chip surface was then regenerated by 60 μl of 2 M NaCl. To correct the nonspecific binding and bulk refractive index change, a blank channel without SPMG (FC2) was run simultaneously for each experiment. The sensorgram from the blank channel was subtracted from the sensorgram of test binding, and the specific binding was recorded as resonance units.

Statistical analyses
Data were expressed as mean values ± standard deviation (S.D.). Student’s t-test and analysis of variance (ANOVA) following by the Newman-Keuls post hoc test were performed to assess differences between the relevant control and each experimental group. P-values of <0.05, <0.01, and <0.001 were regarded as indicating statistical significance.

Results
Intraperitoneal administration of SPMG: enhancement of ConA-induced in vitro proliferation of thymocytes
Information on lymphocyte survival and/or proliferation is the conventional first requirement for evaluation of the immunological functions. Therefore, we firstly detected the influence of SPMG on thymocyte proliferation by MTT assay (Fig. 1). ConA-stimulated thymocytes were remarkably proliferated by 230%, compared with nonstimulated cells. In addition, SPMG at 5 and 10 mg/kg (but not 2.5 and 20 mg/kg) further enhanced ConA-stimulated thymocyte proliferation.

SPMG upregulated cell surface expression of CD69
The expression of surface antigen CD69 in immune response cells is typically associated with the early stage of cell activation, with maximal expression reached at about 4 h by appropriate antigenic or mitogenic stimulation (9). In our study, flow cytometric analysis showed that thymocytes exhibited a remarkable expression of surface CD69 following 6-h stimulation by ConA (28 ± 3.5%) (Fig. 2). SPMG at 5 (39 ± 4.2%) and 10 mg/kg (44 ± 5.3%) enhanced ConA-induced CD69 expression in thymocytes, while SPMG at 2.5 (25 ± 6.4%) and 20 mg/kg (29 ± 6.5%) showed no significant effect.

Fig. 1. Effect of chronic intraperitoneal injection of SPMG in Balb/c mice: enhancement of ConA-induced in vitro proliferation of isolated thymocytes. Five groups of mice (each group, n = 10) received intraperitoneal injection of saline (control group) or the indicated doses of SPMG for 6 weeks; the thymocytes were isolated from each group of mice. The cells (5 x 10^5/well) were cultured without or with 5 μg/ml ConA for 48 h, and the cell proliferation was measured by MTT assay. A value of 1 for the proliferative index represents cell proliferation obtained in ConA-nontreated cells. Mean ± S.D. (n = 10). *P<0.05, **P<0.01 vs control.
SPMG enhanced the ratio of CD4 over CD8 in T lymphocytes

Many studies have shown that drugs with immunomodulating effects always cause changes of lymphocyte subpopulations (15), which thus lead us to investigate the effects of SPMG on the T lymphocyte subset in peripheral blood mononuclear cells (PBMC) (Fig. 3). SPMG at 5 (4.07 ± 0.68) and 10 mg/kg (4.13 ± 0.51) significantly enhanced the CD4/CD8 ratio of T cells, compared with the control group (3.38 ± 0.58); in contrast, SPMG at 2.5 (3.25 ± 0.67) and 20 mg/kg (3.51 ± 0.56) had no significant effect.

SPMG modulated the production of intracellular cytokines in T lymphocytes

Assuming that the immunopotentiating effect of SPMG might be associated with the increased synthesis of cytokines, we examined in CD3+ lymphocytes of PBMC whether SPMG could augment PMA/ionomycin-induced expressions of cytokines, in particular, those of IL-2, IFN-γ, and TNF-α (Fig. 4).

SPMG at 5 and 10 mg/kg (but not 2.5 and 20 mg/kg) significantly increased cellular levels of IL-2 (27 ± 3.7% and 29 ± 4.2%, respectively) and IFN-γ (4.4 ± 0.59% and 5.5 ± 0.89%, respectively), as compared to IL-2 (20 ± 2.3%) and IFN-γ (3.5 ± 0.51%) levels in the control group. In contrast, SPMG at 5 and 10 mg/kg (but not 2.5 and 20 mg/kg) dramatically decreased cellular level of TNF-α (45 ± 5.8% and 40 ± 4.2%, respectively), compared to the control group (56 ± 3.7%).

SPMG interacted with IL-2 at the molecular level

Because cytokines are the pivotal regulators of immune responses, we examined whether SPMG could interact with IL-2, a prominent regulator of the immune system, by using the SPR assay. With SPMG immobilized on the sensor chip surface, IL-2 at 0.33, 0.65, 1.29, 2.58, and 5.16 μM was injected over the sensor chip surface, and the binding of IL-2 to SPMG was measured; IL-2 bound to SPMG, and it was increased, depending on the increasing concentrations of IL-2 (Fig. 5).

In addition, the kinetic studies by BIAcore evaluation 3.0 Software indicated that SPMG bound to IL-2 with a high-affinity (Kd = 9.53 × 10⁻⁸ M) (Table 1).

Stoichiometry of the binding between SPMG and IL-2 was calculated, according to the following formulation
Modulation of T Cell Function by SPMG

Fig. 3. CD4 and CD8 subsets in T cells: enhancement of CD4/CD8 ratio following 6-week-intraperitoneal injection of SPMG. Whole blood samples were stained with CD3-Cy, CD8-FITC, and CD4-PE mAbs and analyzed using flow cytometry. A: CD3^+ cells (R2) were defined by regions on the dot-plots of CD3-Cy fluorescence signals and side scatter signal, B: control, C: 2.5 mg/kg, D: 5 mg/kg, E: 10 mg/kg, F: 20 mg/kg of SPMG. Mean ± S.D. (n = 10). *P<0.05 vs control.

Fig. 4. Production of IL-2, IFN-γ, and TNF-α in the CD3^+ lymphocytes following 6-week intraperitoneal injection of SPMG. Whole blood samples were stimulated with PMA/ionomycin for 6 h. Cells were then stained with CD3-FITC and cytokine-PE mAbs and analyzed using flow cytometry. A: CD3^+ cells (R1) were defined by regions on the dot-plots of CD3-FITC fluorescence signals and side scatter signal, B: control, C: 2.5 mg/kg, D: 5 mg/kg, E: 10 mg/kg, F: 20 mg/kg of SPMG. Mean ± S.D. (n = 10). *P<0.05 vs control.
Where $R_{\text{max}}(\text{IL-2})$ is 2053 RU, $R_{\text{immboSPMG}}$ is 514 RU, $MW_{\text{SPMG}}$ is 8 KDa, and $MW_{\text{IL-2}}$ is 16 KD.

Results indicated that one molecule of SPMG bound to 2 or 3 molecules of IL-2.

It is known that IL-2, like other cytokines, contains the heparin-binding domain in its primary sequence (16, 17). SPMG bears highly negative charges, and highly negative charges interact with IL-2 and other cytokines, thus prompting us to presume that SPMG may share the overlapping binding sites with heparin on the IL-2 molecule. In our present study, IL-2 at 10 μg/ml was preincubated without or with 10 μg/ml heparin at RT for 10 min, and the incubation medium was flowed over the biosensor surface, immobilizing SPMG (Fig. 6); IL-2 alone bound to SPMG in a time-dependent manner, whereas complex formation between IL-2 and heparin reduced interaction between SPMG and IL-2. We then examined whether the same pretreatment of IL-2 with various glycosaminoglycans (GAGs) can also interfere with the SPMG-IL-2 interaction (Table 2); only heparin (but not other GAGs) almost completely blocked the interaction of SPMG with IL-2 by 72.2%, with the resonance units falling from 636.6 RU to 176.9 RU. Thus, SPMG might share common binding sites with heparin on the IL-2 molecule.

**Heterocomplex of SPMG and IL-2 increased thymocytes proliferation in vitro**

The fact that IL-2 binding to SPMG was concentra-
tion-dependent and high-affinity further led us to examine if the heterocomplex formation between SPMG and IL-2 might facilitate the IL-2-induced T cell proliferation by using the MTT assay (Fig. 7). The preincubation of 50 IU/ml IL-2 with SPMG at 1, 10, and 100 μg/ml enhanced thymocyte proliferation, compared with IL-2 alone, with no concentration-dependent stimulating effect of SPMG. In contrast, SPMG alone at 1, 10, and 100 μg/ml failed to stimulate thymocyte proliferation, as compared to SPMG-nontreated cells (data not shown).

Discussion

In activated T cells, one of the earliest markers expressed during immune responses is antigen CD69 (18), a transmembrane protein designated as the activation inducer molecule (AIM) or early activation antigen (EA-1) or Leu-23 (19). Immune response cells normally express very low levels of CD69 in their quiescence, yet rapidly increase their expression of surface CD69 following stimulation by a variety of mitogenic agents (e.g., ConA, IL-2, phorbol esters or TCR engagement) (20). In our present study, flow cytometric analysis demonstrated that SPMG enhanced ConA-induced surface expression of CD69 in T cells, suggestive of the involvement of SPMG in proliferation-promoting activities and subsequent modulation of T cells.

Homeostasis of T lymphocyte subpopulation numbers and percentages is maintained by complex, not fully-understood, regulatory mechanisms (21), but profound disturbances in the normal CD4 and CD8 lymphocyte profile were often observed in certain disease states such as AIDS (22, 23). A large body of evidence has documented that polysaccharides can modulate such disturbed lymphocyte subsets (24). Our finding that SPMG also integrated lymphocyte differentiation and thus significantly altered the ratio of CD4$^+$ over CD8$^+$ in T cell subsets is quite in agreement with such a notion, yet the possible impact of SPMG on the T cell subset remains to be clarified.

It is well acknowledged that many cytokines are released from lymphocytes upon stimulation to regulate different immune responses. This raised possibility that SPMG might exert a profound effect on cytokine release following lymphocyte activation. In our present study, SPMG remarkably increased release of intracellular IL-2 and IFN-γ, which was accompanied by decreased release of intracellular TNF-α. The enhancement of IL-2 and IFN-γ release by SPMG in our present study may provide a new insight into the modulating effects of SPMG on T cell function. At the same time, the mechanisms whereby SPMG decreased expression of intracellular of TNF-α and the impact of TNF-α down-regulation on immunomodulation need further investigation.

Notably, IL-2, IFN-γ, and IL-12 are soluble proteins that readily diffuse into the surrounding tissues immediately after the extracellular secretion; an important question is how the immune responses they stimulate remain focused at the secretion sites of tissues with localized infection. An emerging mechanism favoring paracrine rather than systemic endocrine activation is that a number of cytokines bind to GAGs, in particular heparin and the heparan sulfate. These GAGs are long, unbranched chains of acidic carbohydrate occurring on the cell surface and in the extracellular matrix, rendering binding of GAGs to cytokines (16, 25). Thus, the binding of these cytokines to GAGs is likely to retain cytokines close to their sites of secretion, serving to maintain high local concentrations of these soluble mediators (17). In our present study, SPMG bound to IL-2 with a high-affinity ($K_a = 9.53 \times 10^{-7}$ M). It is of note that heparin remarkably blocked SPMG binding to IL-2 in a competitive manner, implicating that SPMG and heparin shared, to a large extent, common binding sites on the IL-2 molecule; in contrast, other GAGs had little effect on interaction between SPMG and IL-2. It is generally thought that specific recognition for IL-2 requires a defined sugar sequence; however, the interaction of proteins with polysaccharides predominately depends on saccharide composition, polysaccharide size, extent and distribution of sulfation, negatively charged carboxyl groups of the polysaccharide back-
bone, and so on (26, 27). Therefore, we tentatively presumed that the high-affinity interaction between SPMG and IL-2 may be attributable to the defined sugar sequence rather than the sole existence of negatively charged groups in the SPMG molecule. Another important finding in our present study is that preincubation of SPMG with IL-2 enhanced the proliferative effect of IL-2 on lymphocytes. Like the heparin-IL-2 complex, the SPMG-IL-2 complex may permit IL-2 presentation on the cell membrane, thus allowing IL-2 approach closely to its receptor IL-2R. Our data by SPR that SPMG bound to more than one molecule of IL-2 (SPMG:IL-2 = 1:2 or 3) might greatly favor oligomerization of IL-2, thus promoting activity of the SPMG-IL-2 complex on T cell function.

Collectively, we demonstrated for the first time that SPMG exerted its immunopotentiation by direct activation of T cell function, accompanied by simultaneous modulation of cytokine function. The elucidated cytokine-dependent immunopotentiation of SPMG helps us to further understand the potential roles of other polysaccharides, involved in immunomodulating activity and consequent anti-AIDS therapy. All these highlight the distinguished role of SPMG, as compared to other anti-AIDS compounds, and gave us promising evidence for the usefulness of SPMG in AIDS therapy. Notably, it is particularly important for us to evaluate the immunomodulating functions of SPMG in pathological immune responses in the future and especially in AIDS patients during our Phase II clinical trial.

Acknowledgments

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