Regular Articles Miscellaneous

Serotype-specific Modulation of Human Monocyte Functions by Glycopeptidolipid (GPL) Isolated from *Mycobacterium avium* complex

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Immunomodulating activity of glycopeptidolipids (GPL), separated from different serovars of *Mycobacterium avium* complex (MAC), on macrophage functions was compared. When peripheral blood mononuclear cells (PBMC), from healthy donors showing strongly positive reactions to mycobacterial purified protein derivatives (PPD), were incubated with heat-killed *Staphylococcus aureus* coated with GPL serovar 4, phagocytosis of monocytes increased in dose-dependent manner. However, coating with GPL serovar 9 did not show any effects. After phagocytosis of heat-killed *S. aureus*, the phagosome-lysosome (P-L) fusion in monocytes was inhibited dose-dependently by coating of *S. aureus* with GPL serovar 4, but not serovar 9. These results indicate that GPL serovar 4 facilitates invasion of MAC into monocytes and renders resistance to bactericidal reactions due to the inhibition of P-L fusion. Regarding accessory function of macrophages in proliferative responses of T cells, the addition of GPL serovar 4 to cultures resulted in significant inhibition of anti-CD3 monoclonal antibody (mAb)-induced proliferation, whereas both serovar GPLs did not cause reduction of cell viability. Furthermore, the PPD-specific T cell proliferative response was downregulated by GPL serovar 4, but weakly suppressed by GPL serovar 9. These results indicated that the immunomodulating activity of GPL on macrophage functions is serovar-dependent.

Key words: Mycobacteria, MAC, GPL, Macrophage, phagocytosis, proliferative response

Disseminated infection with *M. avium* complex (MAC), a typical intracellular parasite similar to *Mycobacterium tuberculosis*, frequently occurs in the advanced stages of AIDS and reduces the mean survival time compared with that of MAC-free AIDS patients (1). During the long-term infection with MAC, surface lipid components are synthesized and accumulated in macrophages (2-4).

Among lipid components produced by MAC, serotype-specific glycopeptidolipids (GPL), differing from typical glycolipids of *M. tuberculosis* such as trehalose dimycolate and mannose-capped lipoarabinomannan, have immunomodulating activities (5).

Among more than 31 serotypes of MAC reported so far (5), MAC with serovar 1, 4, 8 and 9 were frequently identified in AIDS patients, and the cumulative results

demonstrate that the serovar 4 was predominant in clinical isolates of AIDS patients (1). Stimulation of human peripheral blood mononuclear cells (PBMC) and MonoMAC 6 cells, a human macrophage cell line, with GPL serovar 4 induced production of cytokines TNF-α, IL-1β and IL-6 as well as lipid mediators prostaglandin E_2 and thromboxane B_2 (6, 7). When human PBMC were exposed for 24 h to GPL, especially GPL serovar 4, prior to treatment with phytohemagglutinin and phorbol myristate acetate, Thl cytokine production such as IL-2 and IFN-γ was significantly suppressed (8). In addition, intraperitoneal injection with GPL to mice downregulated mitogen-induced proliferative responses of spleen cells (9).

From the structural analyses, the GPL share a common core structure (5, 10), as shown in Fig. 1. Tassell suggested that the common core part of GPL seemed to play an important role in expression of immunomodulating activity (11). However, little study has been performed regarding the modulating activity of GPL on macrophage functions. Therefore, we examined the activity of typical serovar 4 and 9 GPL, frequently found in clinical isolates, on macrophage functions.

MATERIALS AND METHODS

Preparation of heat-killed MAC and GPL. *M. avium* serovar 4 and 9, clinical isolates, were cultivated in Middlebrook 7H9 broth (Becton Dickinson Microbiology Systems, Spark, MD, USA) with glycerol (2 ml/L) and Middelebrook ADC Enrichment (100 ml/L) at 37 °C for 3 weeks, killed by autoclaving and obtained as heat-killed bacilli after washing with PBS. The bacilli were treated with 30-fold volume of $CHCl_3$: CH_3OH (2: 1, v/v). After removing delipidated bacilli, a lipid fraction obtained was treated with 0.2 N NaOH in methanol 37 °C for 90 min and a resultant alkaline-resistant lipid fraction was recovered. GPL was isolated from the lipid fraction using UNIPLATE™ TLC plates (ANALTECH Inc., Newark, DE, USA) with $CHCl_3$: CH_3OH: H_2O (65: 25: 4, v/v/v). The purity was confirmed by TLC.

Preperation of heat-killed *Staphylococcus aureus* and coating with GPL. *S. aureus* 209P was cultivated on Heart Infusion Agar (Becton Dickinson Microbiology Systems) 37 °C for 24 h, and bacteria obtained were suspended in 0.1% gelatin-Hanks' balanced salt solution (HBSS; Nissui Pharmaceutical Co., Tokyo) and then killed by autoclaving. To prepare GPL-coated bacilli, heat-killed *S. aureus* (1 x 10^8 cells) ware mixed with 0.2 to 25 μg of either GPL of serovar 4 or 9 in methanol, and the solvent was removed by evaporating. GPL-coated bacilli were suspended in 1 ml of 0.1% gelatin-HBSS and sonicated well.

Peripheral blood mononuclear cells (PBMC). Heparinized blood samples were taken from healthy donors showing strongly positive reaction to mycobacterial PPD, mixed with equal volume of Dulbecco's phosphate buffered saline (PBS), and layered on Ficoll-Paque™ PLUS (Amersham Pharmacia Bioteck, Uppsala, Sweden). After centrifugation for 30 min at 400 x g, mononuclear cells were recovered from the interphase, suspended in RPMI 1640 medium (Nissui Pharmaceutical Co.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen Corp., Carlsbad, CA, USA), 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol, and adjusted at a cell number of 1 x 10^6 /ml.

Phagocytosis. Aliquots (0.2 ml) of PBMC suspensions (5 x 10^6/ml) were distributed on the 13-mm round cover slip and incubated for 90 min at 37 °C. After removing non-adherent cells, aliquots (0.2 ml) of bacterial suspensions (1 x 10^8/ml) were added to adherent cultures and incubated for 3 h at 37 °C. After washing three times with PBS, cells were fixed with methanol and stained with methylene blue. Phagocytosis by monocytes was estimated microscopically. Results show as phagocytosis index, which is calculated from the following formula: phagocytosis index = (total

number of bacteria phagocytosed /total number of monocyte) x (total number of monocytes phagocytosed bacteria/total number of monocyte) x 100.

Measurement of phagosome-lysosome (P-L) fusion. Monocytes adhered to cover slips, prepared as described above, ware incubated in the presence of 5 μg/ml acridine orange solution (Sigma-Ardrich Co., St. Louis, MO, USA) for 15 min 37 °C. After washing with PBS, GPL-coated *S. aureus* bacilli (1 x 10^8) were put on labeled monocytes and incubated for 3 h at 37 °C. After washing three times and drying, numbers of acridine orange-stained bacilli were enumerated. Results represent as fusion index, which is calculated from the following formula: Fusion index = (total number of bacteria P-L fused /total number of monocyte) x (total number of monocyte P-L-fused /total number of monocyte) x 100.

Proliferative response of T cells. Aliquots (50 μl) of GPL serovar 4 and 9 in ethanol ware distributed to 96-well plates and evaporated overnight. PBMC (1 x 10^5) in a volume of 0.1 ml were incubated in the presence of GPL, coated as a solid phase on wells, followed by the stimulation with either 0.1 μg anti-CD3 (OKT-3) mAb or 2 μg PPD for 3 days. Eight hours before termination of incubation, aliquots (0.5 μCi) of [methyl-^{3}H] thymidine (specific activity: 925 GBq/mmol, Amersham Bioscience) were added to the well. The radioactivity of tritiated thymidine incorporated into T cells was measured by a liquid scintillation counter (Wallac, Turk, Finland).

Determination of viability and cytotoxicity. Influence of GPL on cell viability of PBMC stimulated with anti-CD3 mAb was estimated by absorbance at 450 nm when WST-1 reagent (Dojindo, Kumamoto) was added to cultures 3 h before termination. Cytotoxicity was estimated by levels of lactate dehydrogenase (LDH) released into culture supernatants. Serially diluted test samples (25 μl) were mixed with equal volume of the Toxicology assay kit (Sigma-Aldrich) stood at room temperature for 20 min and estimated absorbance at 492 nm (reference at 630 nm).

Statistic analysis. Statistical significance of the data was determined by Student's *t*-test. A *P* value of less than 0.05 was taken as significant.

RESULTS

Enhancement of phagocytosis by GPL serovar 4 but not 9. Since MAC is a typical intracellular parasite similar to *M. tuberculosis*, phagocytosis by macrophage is one of the convenient invasion systems. Human PBMC were incubated in the presence of GPL-coated *S. aureus* and numbers of bacteria phagocytosed by monocytes were enumerated microscopically. The phagocytosis index increased in a dose dependent manner when bacteria were coated with GPL serovar 4 but not 9 (Fig. 2). A significant increase was detected over 1 μg/ml. This result indicates that GPL serovar 4 is the important lipid for facilitating invasion of MAC.

Inhibition of the P-L fusion in human monocytes by GPL serovar 4 but not 9. The intracellular parasite such as *M. tuberculosis* can escape from the killing mechanisms of macrophages by which *M. tuberculosis* inhibits the P-L fusion followed by suppression of release of various bactericidal molecules (12, 13). Human PBMC were incubated in the presence of GPL-coated *S. aureus* and the number of monocyte with the P-L fusion were enumerated microscopically. Fusion index significantly decreased dose-dependently when bacteria coated with GPL serovar 4 but not 9 were added (Fig. 3). This result indicates that GPL serovar 4 is the important lipid for escape of MAC from killing mechanisms.

Inhibition of anti-CD3-stimulated proliferative response of T cells by GPL serovar 4. Proliferative response of T cells needs accessory functions of macrophages. To estimate whether GPL serovar 4 affects accessory functions of macrophages, proliferative responses

were measured in the presence or absence of GPL serovar 4 when T cells in PBMC were stimulated with anti-CD3 mAb coated on wells. As shown in Fig. 4, tritiated thymidine uptake into T cells was significantly suppressed at a dose of 25 and 50 μg/ml. The activity of GPL serovar 9 was very weak at a dose of 50 μg/ml (data not shown). To clarify whether the suppression of proliferative response by GPL serovar 4 is due to the cytotoxicity, viability and cytotoxicity were determined by WST-1 and LDH release, respectively. The addition of both GPL serovar 4 and 9 to cultures did not reduce viability (Table 1). GPL serovar 9 showed a significant cytotoxic activity but GPL serovar 4 did not.

Inhibition of antigen-specific T cell response by GPL serovar 4. but not 9. Since GPL serovar 4 but not 9 suppressed anti-CD3-induced proliferative response, we examined that GPL can suppress antigen-specific T cell response. PBMC, from healthy donors showing the strong positive reaction to PPD, were stimulated with PPD in the presence of 50 μg/ml GPL serovar 4 and 9. GPL serovar 4 significantly suppressed PPD-specific proliferative response, but GPL serovar 9 did not. This result indicates that GPL serovar 4 suppresses the T cell function, which plays a pivotal role in adaptive immunity against MAC infection, by modulating accessory function of macrophages, and that the MAC serovar is critical for expression of virulence.

DISCUSSION

Opportunistic infection with MAC frequently occurs in the advanced stage of AIDS and reduces the mean survival time (1). The present study demonstrated that GPL serovar 4, a characteristic lipid of MAC virulent strain, was able to suppress both innate and adoptive immunity. That is, not only inhibition of the P-L fusion in phagocytosis of bacteria by neutrophils and macrophages is one of the representative defense mechanisms. Interestingly, phagocytosis of *S. aureus* by human monocytes was enhanced, when *S. aureus* were coated with GPL serovar 4, but not serovar 9, indicating that a type of GPLs present on the cell surface of MAC accelerates their invasion into phagocytes. Indeed, GPLs from MAC serovar 5, 7 and 12 exhibited similar activity in terms of enhancement of phagocytosis. Another type of GPLs was also present, and GPL serovars 6, 8, 10, 13 and 14 did not enhance phagocytosis (unpublished data). Regarding the P-L fusion to degrade phagocytosed bacteria, GPL serovar 4 inhibited the fusion in human monocytes. GPL serovar 12 also showed similar activity. By contrast, GPL serovars 8, 10, 13 as well as serovar 9 did not inhibit the fusion (unpublished data). Further experiments need for clarifying the mechanism of GPL in invasion of MAC into cells and escape from bacterial killing.

In addition to modulation of phagocytosis by GPL, we demonstrated that treatment with GPL serovar 4, but not serovar 9, resulted in modulation of other macrophage functions since anti-CD3-induced and PPD-specific T cell proliferative responses were significantly suppressed. This result indicates that T cell responses in adoptive immunity are also modulated by treatment with GPL serovar 4, and suggests that infection with MAC serovar 4 causes more severe conditions in terms of defense ability of AIDS patients against pathogens. Mitogen-induced Th1 cytokine production such as IL-2 and IFN-γ was significantly suppressed in human PBMC by pretreatment with GPL serovar 4 (8). By contrast, GPL from MAC serovar 4 caused production of bioactive components such as cytokines TNF-α, IL-1β and IL-6 as well as lipid mediators prostaglandin E_2 and thromboxane B_2 (6,7). These results suggest that further experiments are needed to clarify the activity of GPL on production of cytokines, chemokines and lipid mediators.

As shown in Fig. 1, the core of GPL is shared a common structure but the serotype-specific oligosaccharides, linked

to allo-threonine in the core, are different. Therefore, the different activity of GPLs among the serotypes in modulation of macrophage functions would be due to the structural difference of oligosaccharide part of GPLs. On the other hand, the acyl group in the core is not simple. In our preliminary study on GPL serovar 7, the acyl groups not only composes of linear C16 to C20 fatty acids but also of 3-O-methyl C28 to C34 fatty acids (mainly C32). Although we have not yet started the analysis of fatty acid compositions of GPL serovar 4 and 9, the structure-activity relationship of fatty acids in the core is very interesting.

In conclusion, a GPL from virulent strain MAC serovar 4 modulates macrophage function in both innate and adoptive immunity. We are now considering the possibility of a new drug for treating autoimmune diseases.

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Table 1 Inhibition of anti-CD3-induced proliferative response of T cells is not responsible for a decrease of cell viability and an increase of cytotoxicity

(A)	Viability (%) (WST-1)		
Donors	Control (solvent)	with GPL serovar 4	with GPL serovar 9
#1	100 ± 2.4	109 ± 11	139 ± 10
#2	100 ± 1.0	165 ± 12	195 ± 10

(B)	Cytotoxicity (%) (LDH release)		
Donors	Control (solvent)	with GPL serovar 4	with GPL serovar 9
#1	13.2 ± 1.5	8.7 ± 4.7	35.4 ± 9.8
#2	7.7 ± 3.2	14.2 ± 2.7	44.5 ± 6.6

Plates were coated with solvent (ethanol) alone or solvent containing GPL serovar 4 and 9 before stimulation with anti-CD3 mAb. (A) Cell viability (%) was estimated by absorbance at 450 nm when WST-1 reagent (Dojindo, Kumamoto) was added to cultures 3 h before termination. (B) Cytotoxicity (%) was estimated by the levels of lactate dehydrogenase (LDH) released into culture supernatants using Toxicology assay kit (Sigma) (absorbance at 492/ 630 nm).

Oligosaccharide (variable)

Oligosaccharide

Serovar 4

Serovar 9

Fig. 1 Structure of GPL.

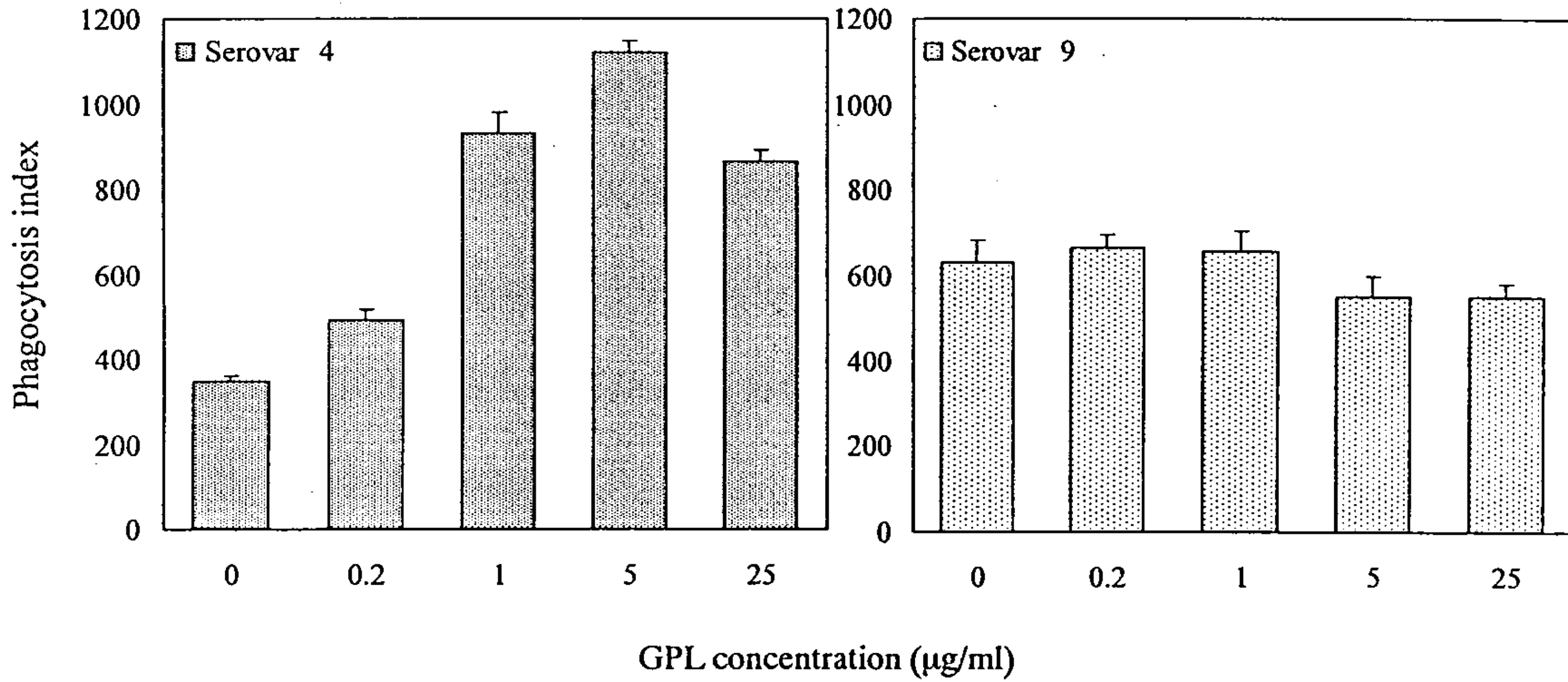

Fig. 2 Enhancement of phagocytosis by GPL serovar 4 but not serovar 9.
Human PBMC (5 x 10^6/ml) were stimulated with indicated amount of GPL-coated *S. aureus*. Three hours after the stimulation, cells were fixed with methanol and stained with methylane blue. Phagocytosis by monocytes was estimated microscopically. Results are shown as phagocytosis index, which is calculated from the following formula: phagocytosis index = (total number of bacteria phagocytosed /total number of monocytes) x (total number of monocytes phagocytosed bacteria/total number of monocyte) x 100

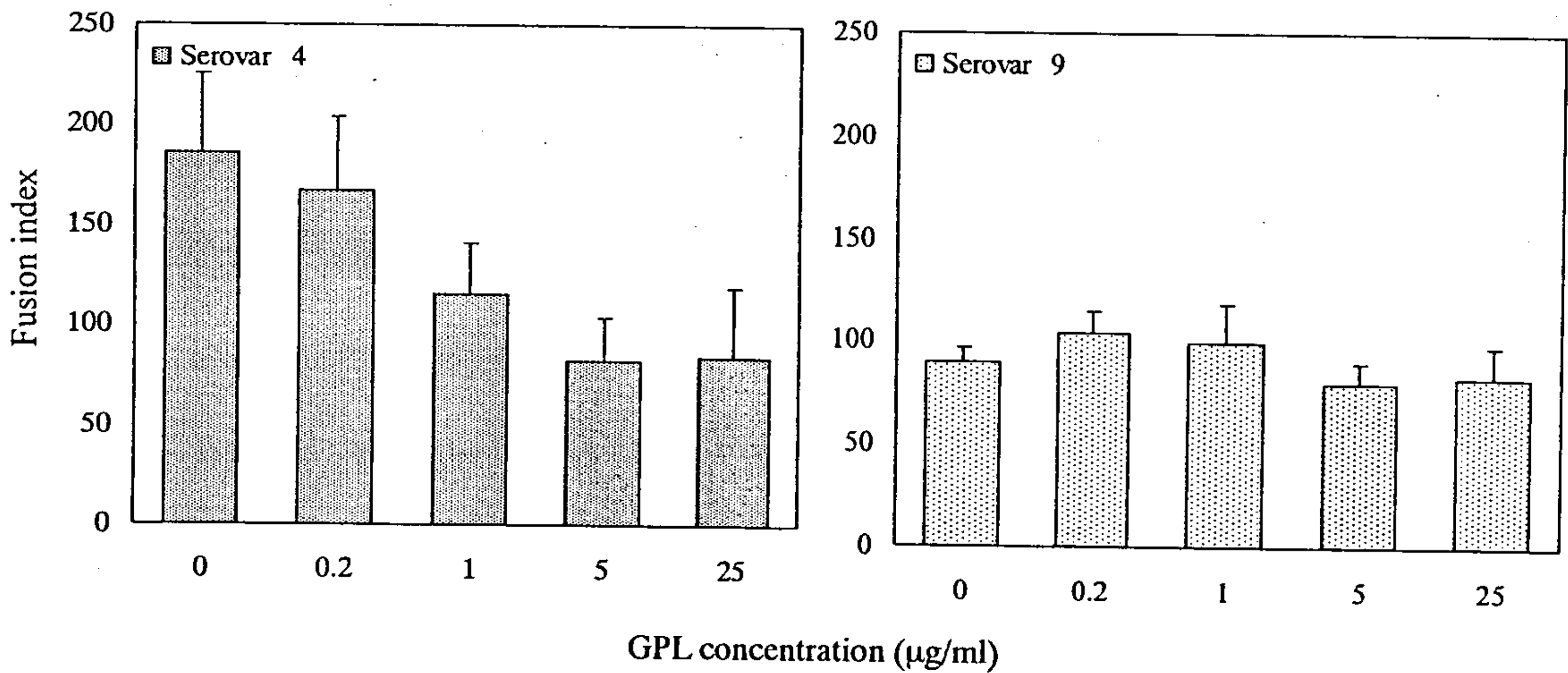

Fig. 3 Inhibition of P-L fusion by GPL serovar 4 but not serovar 9.
Human PBMC (5 x 10^6/ml) were incubated in the presence of 5 μg/ml acridine orange solution for 15 min. After washing, labeled cells were stimulated with indicated amount of GPL-coated *S. aureus*. Three hours after the stimulation, after washing three times and drying, number of acridine orange-stained bacilli were enumerated. Result are shown as fusion index, which is calculated from the following formula: Fusion index = (total number of bacteria P-L fused /total number of monocyte) x (total number of monocyte P-L-fused /total number of monocyte) x 100.

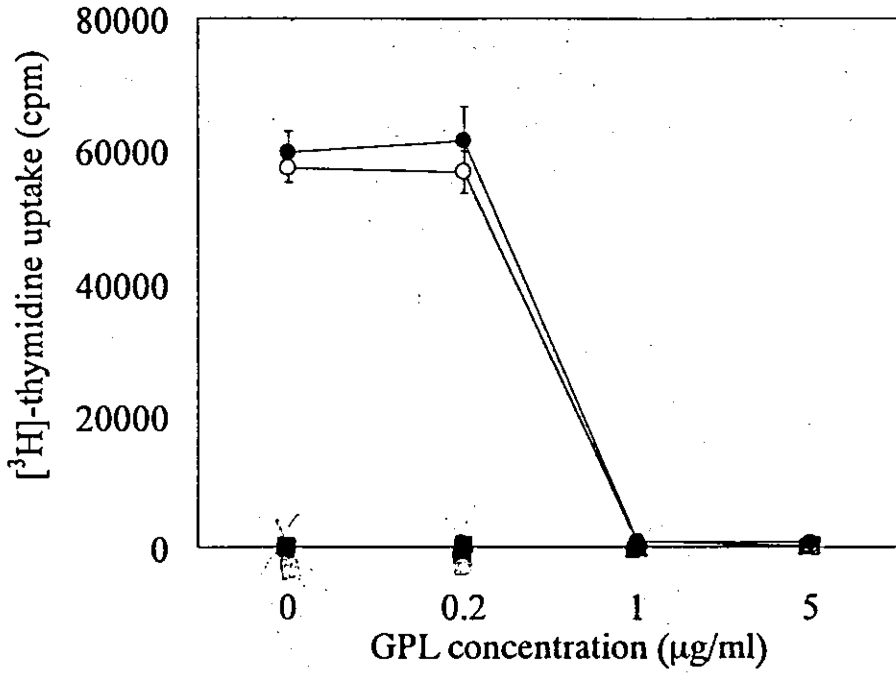

Fig. 4 Inhibition of anti-CD3-stimulated proliferative response of T cells by GPL serovar 4.
Human PBMC (5 x 10^6/ml) were incubated in the presence of GPL, coated as a solid phase on wells, followed by the stimulation with 0.1 μg anti-CD3 (circle) or medium alone (square) for 3 days. Eight hours before termination of incubation, aliquots (0.5 μCi) of [methyl-^{3}H] thymidine were added to the well. The radioactivity of tritiated thymidine incorporated into T cells was measured by a liquid scintillation counter. Open and closed symbols represent different donors.

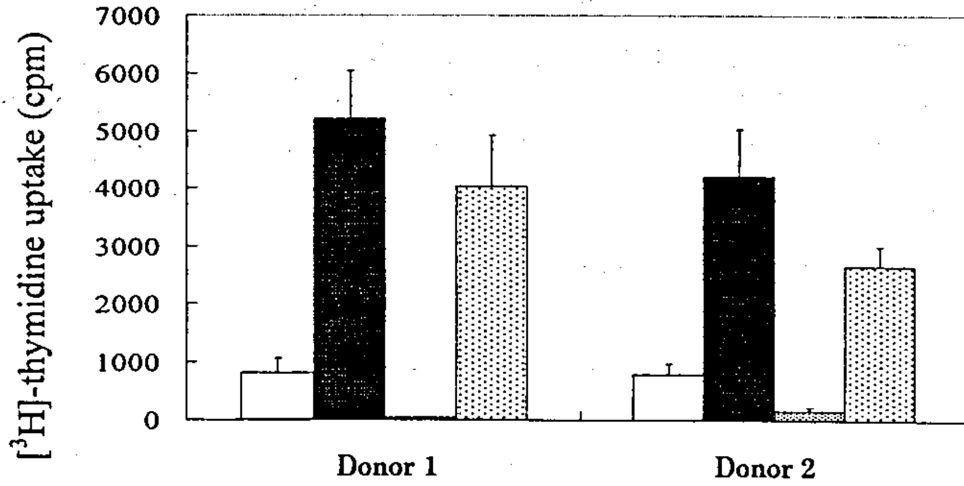

Fig. 5 Inhibition of antigen-specific T cell response by GPL serovar 4 but serovar 9.
Human PBMC (5×10^6/ml) were incubated in the presence of GPL serovar 4 (▒), GPL serovar 9 (░) or solvent (▓) coated as a solid phase on wells, followed by the stimulation with 100 μg/ml PPD for 3 days. Eight hours before termination of incubation, aliquots (0.5 μCi) of [methyl-^{3}H] thymidine were added to the well. The radioactivity of tritiated thymidine incorporated into T cells was measured by a liquid scintillation counter.