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A Monoclonal Antibody to GPI-80, a Novel β_2 Integrin-Associated Glycosylphosphatidylinositol Anchored Protein, Selectively Enhances Macropinocytosis in Human Monocytes

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ABSTRACT

We have shown previously that neutrophils and small population of human monocytes have a glycosylphosphatidylinositol (GPI) -anchored protein, GPI-80 on their surface. In a study of neutrophils, we reported that GPI-80 regulates the adherence and migration. In this study, we examined the functions of GPI-80 in monocytes. Treatment of monocytes with monoclonal antibody (mAb) to GPI-80 enhanced pinocytosis of fluorescein isothiocyanate (FITC)-labeled dextran but not that of lucifer yellow and low-density lipoprotein. This treatment neither changed phagocytosis, adherence nor reactive oxygen production of monocytes. These results suggest that GPI-80 modulates fluid phase macropinocytosis of high molecular weight molecules, but not that of low molecular weight ones, clathrin-dependent pinocytosis, phagocytosis, adherence and reactive oxygen production. Treatment of neutrophils with mAbs to GPI-80 did not change macropinocytosis of FITC-labeled dextran, suggesting that the functions of GPI-80 on macropinocytosis of high molecular weight molecules are different between neutrophils and monocytes. This study, first reported GPI-80 function on monocytes, implies that GPI-80 may be one of the molecules associated with macropinocytosis on monocytes.

Key words : macropinocytosis, monocytes, GPI -anchored protein

INTRODUCTION

GPI-80, a protein mainly expressed on human neutrophils, is a novel member of a β_2 integrin-associated glycosylphosphatidylinositol (GPI)-anchored family of proteins[1)-3)], such as urokinase-type plasminogen activator re-

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ceptor (uPAR), lipopolysaccharide binding protein receptor (CD14), and Fcγ rceptor IIIb[4]. Previous studies have shown that GPI-80 and Vanin-1 (originally reported as a GPI-anchored protein, involved in thymus homing[5]) belong to a large family that is related to amidohydrolase[2),5)-7)]. Published studies by our laboratory demonstrated that GPI-80 is located on microvillous projections of neutrophils and clustered on the forward surfaces of transmigrated neutrophils[8]. Treatment with anti-GPI-80 monoclonal antibody (mAb) (designated 3H9) biphasically modulates β_2 integrin-dependent cell adherence and modulates transendothelial neutrophil migration and locomotion[1), 2), 9)]. The crosslinking of GPI-80 induces tyrosine phospholyration of an unidentified 34-kDa protein[10], up-regulation of CD11b/CD18 (Mac-1) expression on neutrophil surfaces, and shedding of L-selectin, which depends on tyrosine phospholyration and cytoskeleton remodeling[11], suggesting the growing evidence that the molecule may play an important role(s) in the process of β_2 integrin-dependent leukocyte adhesion and locomotion (for review, see[12),13)]). In addition, a recent our study of GPI-80 expression in hematopoietic cells showed a weak immunoreaction on monocytes with 3H9 but not on other hematopoietic cells except neutrophils[1),14)]. We also showed that major GPI-80 positive monocytes belong to a strongly CD14 positive ($CD14^{++}$) subpopulation[15], superior in phagocytosis and reactive oxygen production[16].

During the process of immune reactions, monocytes show an activity of endocytosis, one of the most important cellular functions observed in monocytes. The cellular event is classified into two issues, a constitutive fluid phase endocytosic pathway (pinocytosis), and a phagocytosis of large particles[17]. Pinocytosis is further divided into various pathways involving clathrin-coated vesicle, non-coated vesicle, caveoles or macropinosomes[18]. Reactive oxygen production is another important function in bactericidal functions of monocytes[19]. The aim of the present study is to study possible role(s) of GPI-80 on the surface of monocytes for cellular functions of monocytes through examining the effect of 3H9 on the cellular functions such as endocytosis or reactive oxygen production. Potential role(s) of the GPI-80 on monocyte are discussed.

MATERIALS AND METHODS

Reagents and mAbs

Fluorescein isothiocyanate (FITC)-dextrans (FD) MW 70,000 and 2', 7'-Dichlorofluorescein diacetate (DCFH-DA) were purchased from DAKO (Glostrup, Denmark). DiI-tagged low-density lipoprotein (DiI-LDL), lucifer yellow CH potassium salt (LY), *E.coli* bioparticles fluorescein conjugate and *S.aureus* bioparticles fluorescein conjugate from Molecular Probes (Eugene, OR), RPMI-1640 medium from Life Technologies GibcoBRL (Grand Island, NY), and FCS from ICN Pharmaceuticals, Inc (CostaMesa, CA). All other reagents were of the highest grade commercially available. Preparation of anti-GPI-80 mAb (3H9; mouse IgG1) and FITC-labeling were carried out as described previously[2]. Anti human HLA-A, B, C mAb (MB40.5; mouse IgG1) was purchased from ATCC (Rockville, MD).

Preparation of leukocytes

Leukocytes were isolated from heparinized whole blood from healthy donors using dextran sedimentation as described previously[2]. Neu-

trophils and mononuclear cells (MNC) were prepared using Ficoll-Paque, and then erythrocytes were removed by hypotonic lysis. Cells were suspended in RPMI-1640 containing 10% FCS (culture medium) and used for each experiment.

Flow cytometry

Stained cells were analyzed on FACS Calibur using the Cell Quest program (Becton-Dickinson, Immunocytometry Systems, San Jose, CA). At least 10,000 cells per samples were analyzed. The percentage of positive cells was analyzed from specific and control staining. The data was analyzed using Cellquest program.

Quantitation of pinocytosis

Quantitation of pinocytosis was examined according to the published method[20]. Briefly, suspended leukocytes (1×10^6) were incubated at 37 ℃ for various time periods with 1 mg/ml FD 70,000, LY or 10 μg/ml DiI-LDL. The mixtures were also incubated in the absence or presence of mAbs in 500 μl culture medium. After washing with PBS containing 0.1% BSA and 0.05% sodium azide, monocytes were gated with forward scattered (FSC) and side scattered (SSC) dot plots and fluorescence intensity of gated monocytes was analyzed using FACSCalibur. FD macropinocytosis of neutrophils was also measured with the same methods.

Quantitation of Phagocytosis

Quantitation of phagocytosis on monocytes was determined by previously described methods[21]. Briefly, leukocytes (1×10^6) in 200 μl culture medium were incubated with various concentrations of FITC-*E.coli* or FITC-*S.aureus*. The mixtures were also incubated in the absence or presence of 10 μg/ml mAbs at 37 ℃ for various time periods. After washing twice, monocytes were gated and fluorescence intensity was analyzed.

Measurement of reactive oxygen production

Detection of reactive oxygen production was performed by previously described method[19]. In brief, suspended leukocytes (1×10^7 cells) in 10 ml culture medium were preincubated with 5 μM DCFH-DA with horizontal agitation for 15 min at 37 ℃. After preincubation, 1×10^6 cells were incubated in the presence or absence of 100 nM phorbol 12-myristate 13-acetate (PMA). Cells were also incubated in 10 μg/ml various mAbs without PMA. Incubations were performed for various time periods with horizontal agitation at 37 ℃. After washing, fluorescence intensity was analyzed using a FACS Calibur. Monocytes were gated with FSC and SSC dot-plots, and then each fluorescence intensity was analyzed.

Statistical analysis

Statistical analysis to define significant difference between mean fluorescence intensity (MFI) of pinocytosed FD of 3H9 and control mAb (MB 40.5) treated group in macropinocytosis experiment was performed using Student's unpaired t-test. A probability of $p<0.05$ was considered statistically significant. Data are presented as mean ± SE.

RESULTS

Enhancement of FD macropinocytosis on human monocytes by the treatment with 3H9, but not LY macropinocytosis and clathrin-dependent pinocytosis of LDL

In order to investigate the possible function of GPI-80 in monocytes, we examined the effect of an anti-GPI-80 mAb (3H9) on monocyte functions. Initially, we investigate the effect of 3H9 on endocytosis system of

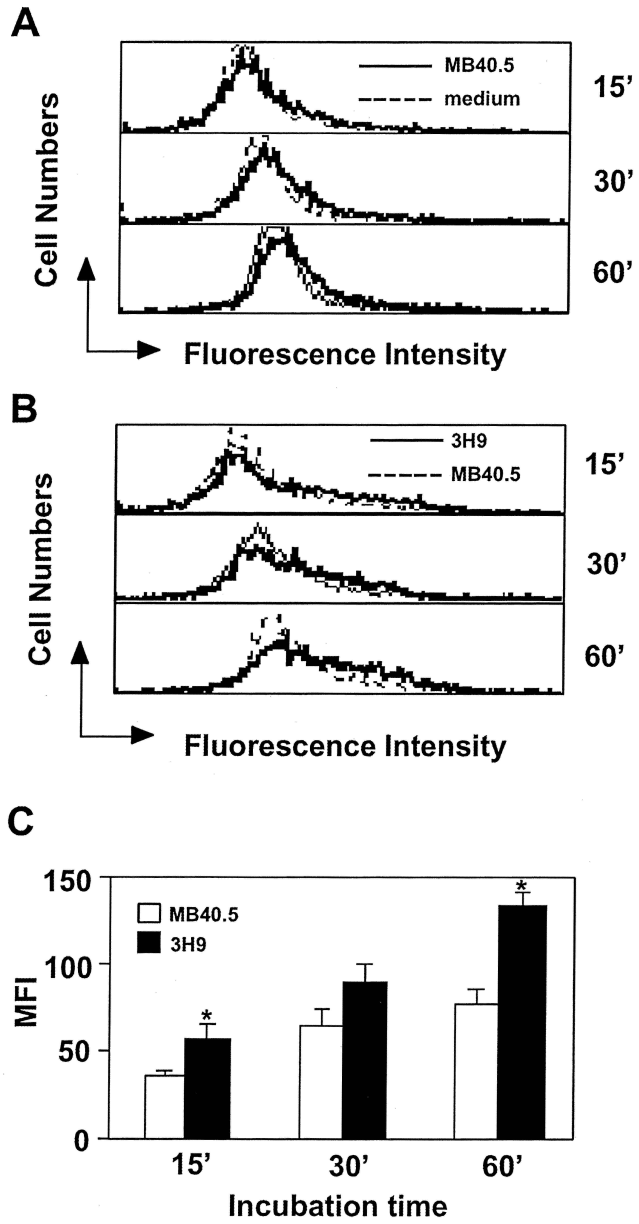

Fig. 1.
Monocyte macropinocytosis of a high MW probe, FD after treatment with 3H9.
Leukocytes were incubated with 1 mg/ml FD 70,000 in the absence (....) or presence (_) of 10 μg/ml MB40.5 (A). The data in the presence of 10 μg/ml MB40.5 (....) or 3H9 (_) (B). In all experiments, the mixtures were incubated for 15, 30 and 60 min at 37 ℃. Monocytes were gated and analyzed for fluorescence intensity. The result showing a representative of six independent experiments. MFI of pinocytosed FD of monocytes, 3H9 treated and control mAb (MB 40.5) treated (C). Data are presented as mean ± SE. of six independent experiments. Significantly different with control mAb treated cells at the same incubation time point. *$p<0.05$

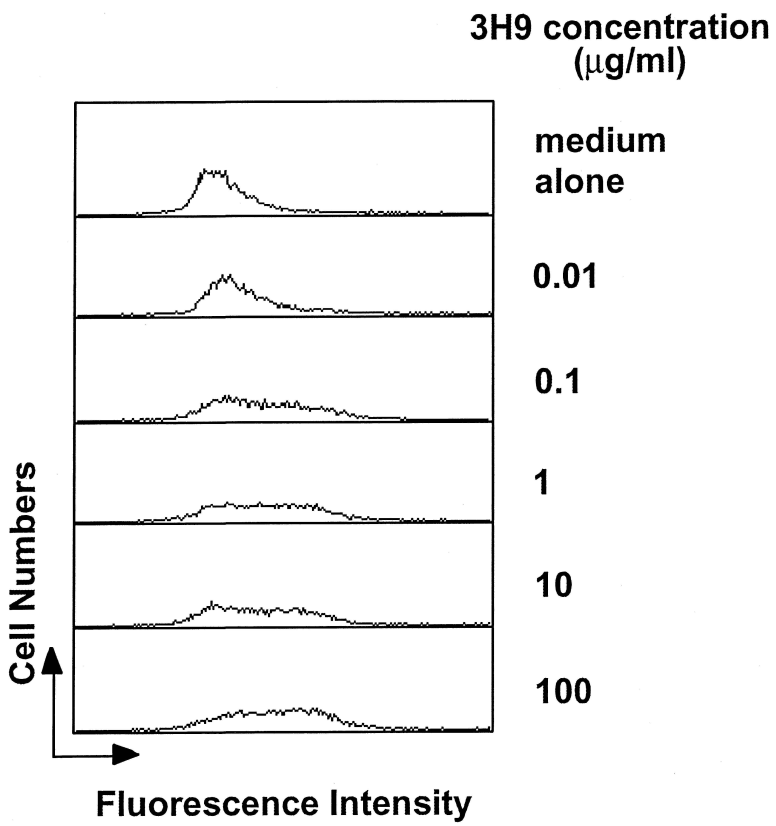

Fig. 2.
Concentration response of 3H9 on monocyte macropinocytosis.
Leukocytes were incubated for 1h at 37 ℃ with 1 mg/ml FD 70,000 in the absence or presence of various concentrations of 3H9 (0.01 μg/ml to 100 μg/ml) in RPMI-1640 medium containing 10% heat inactivated FCS. Monocytes were gated and analyzed for fluorescence intensity. The result is a representative of three experiments.

monocytes. One of the endocytosis pathways, macropinocytosis was evaluated using FD pinocytosis assay. After incubation of monocytes with FD, fluorescence intensity of these cells did not show any changes in the presence of a control antibody MB40.5 (Fig. 1A), whereas a positive shift of the intensity was observed when they were cultured with 3H9 (Fig. 1B). Quantitative analysis of the calculated MFI from the results of Fig. 1B revealed that the MFI was increased in parallel with incubation periods, and the value at 15' and 60' was statistically significant ($p<0.05$) (Fig. 1C). This enhancement of FD macropinocytosis was blocked by wortmannin and cytochalasin D (data not shown). In the concentration response experiment, 3H9 enhanced FD macropinocytosis depending on dose and the treatment of 3H9 reached the maximum effect at the concentration of 0.1 μg/ml and more (Fig. 2).

Attempts were then made to examine the effect of 3H9 on low MW macropinocytosis using LY. Although the LY macropinocytosis of

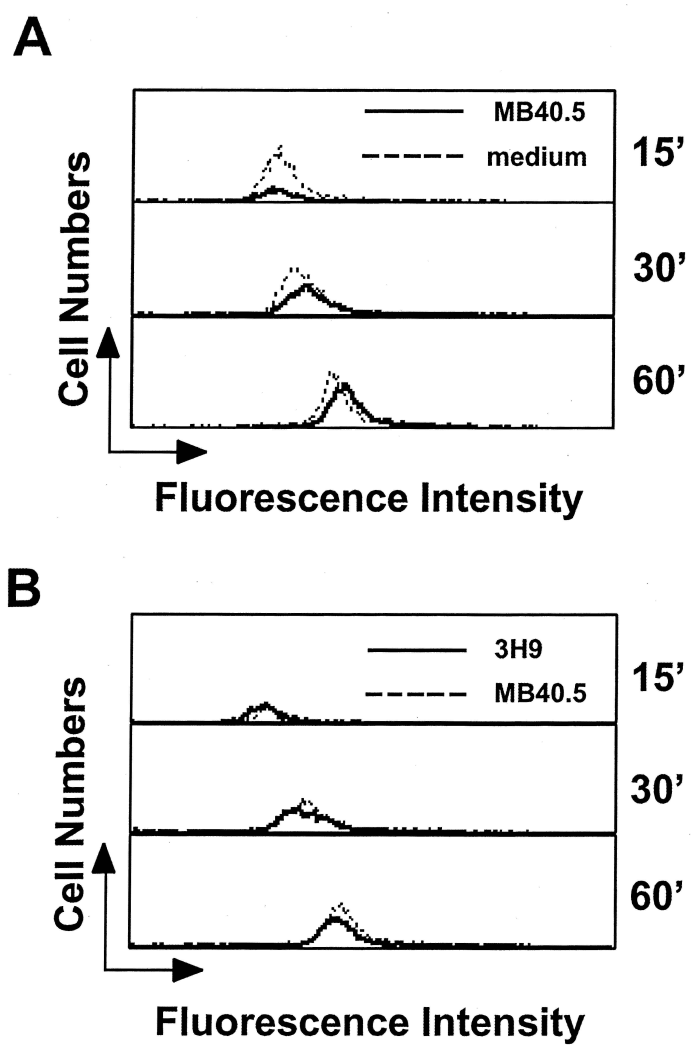

Fig. 3.
Monocyte macropinocytosis of a low MW probe, LY in the presence of 3H9.
Leukocytes were incubated with 1 mg/ml LY in the absence (....) or presence (_) of 10 μg/ml MB40.5 (A), or in the presence of 10 μg/ml MB40.5 (....) or 3H9 (_) for 15, 30 and 60 min at 37 ℃ (B). Monocytes were gated, and the fluorescence intensity of LY was analyzed as described previously. The result is a representative of four experiments.

monocytes increased during incubation periods, the 3H9 did not affect the endocytosis (Fig. 3A and B).

To make clear the effect of 3H9 on another pinocytosis pathway, clathrin-coated pinocytosis on monocytes was measured using DiI-LDL. Although DiI-LDL pinocytosis was spontaneously increased during incubation (Fig. 4A), no effect was observed in the process of the endocytosis by the treatment of 3H9 (Fig. 4B).

Effects of anti-GPI-80 mAbs on phagocytosis,

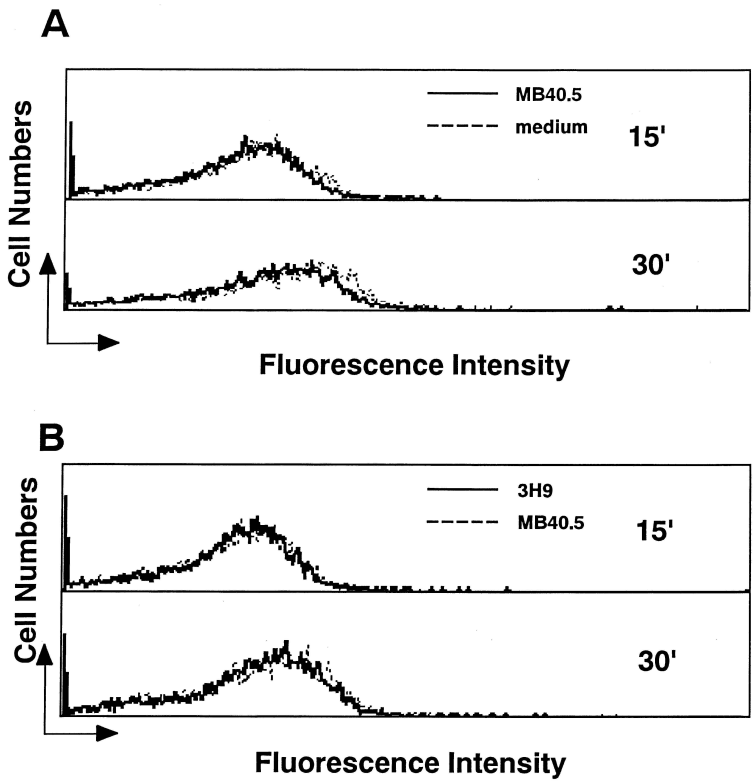

Fig. 4.
Monocyte clathrin-dependent pinocytosis of a DiI-LDL.
Suspended leukocytes were incubated with 100 μg/ml DiI-LDL in the absence (....) or presence (_) of 10 μg/ml MB40.5 for 15 and 30 min at 37 ℃ (A). The data in the presence (....) of 10 μg/ml MB40.5 or 3H9 (_) for 15 and 30 min at 37 ℃ (B). Fluorescence intensity of monocytes was analyzed. The result shown is a representative of three experiments.

adherence of monocytes and reactive oxygen production

To investigate the effect of 3H9 on another endocytosis pathway, i.e., phagocytosis, phagocytised FITC-labeled *S.aureus* in monocytes was analyzed using flowcytometry. As a control study, the increased fluorescence intensity was confined depending on the dose of bacteria concentrations (Fig. 5A). A similar result was obtained using FITC-labeled *E.coli* (data no shown). In contrast, the treatment of 3H9 did not affected phagocytosis, similar to treatment of control mAb (Fig. 5B and C). As a next trial, we examined the monocyte adherence after the 3H9 treatments, because we previously demonstrated that neutrophil adherence was regulated by 3H9 treatment[1),2),9)]. Unexpectedly, 3H9 did not significantly affect the adherence of human monocytes (data not shown). Finally, we analyzed the effect of 3H9 on reactive oxygen production of monocytes. Stimulation of monocytes with PMA was induced into up-regulation of DCFH-DA fluorescence intensity in each incubation periods, suggesting that monocytes produced reactive oxygen production in response to PMA (Fig. 6A). While, 3H9

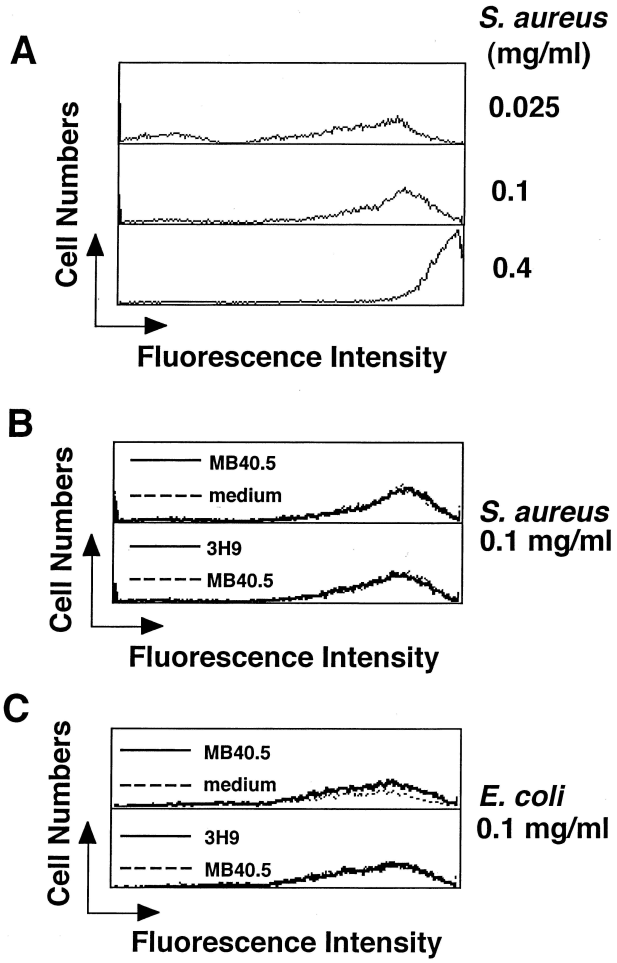

Fig. 5.
Effect of 3H9 on FITC-*S.aureus* or FITC-*E.coli* phagocytosis by monocytes.
Leukocytes were incubated for 1 h at 37 ℃ with increasing concentrations of FITC-*S.aureus*. The fluorescence intensity of FITC-*S.aureus* on monocytes was analyzed using flow cytometry (A). Leukocytes were incubated with various conditions as follows: with 0.1 mg/ml FITC-*S.aureus* in the absence (....) or presence (_) of 10 μg/ml MB40.5, with 0.1 mg/ml FITC-*S.aureus* in the presence (....) of 10 μg/ml MB40.5 or 3H9 (_) (B), with 0.1 mg/ml FITC-*E.coli* in the absence (....) or presence (_) of 10 μg/ml MB40.5, with 0.1 mg/ml FITC-*E.coli* in the presence (....) of 10 μg/ml MB40.5 or 3H9 (_) (C). In all experiments, the mixtures were incubated in culture medium for 60 min at 37 ℃. Monocytes were gated and analyzed for fluorescence intensity. The result is a representative of three experiments.

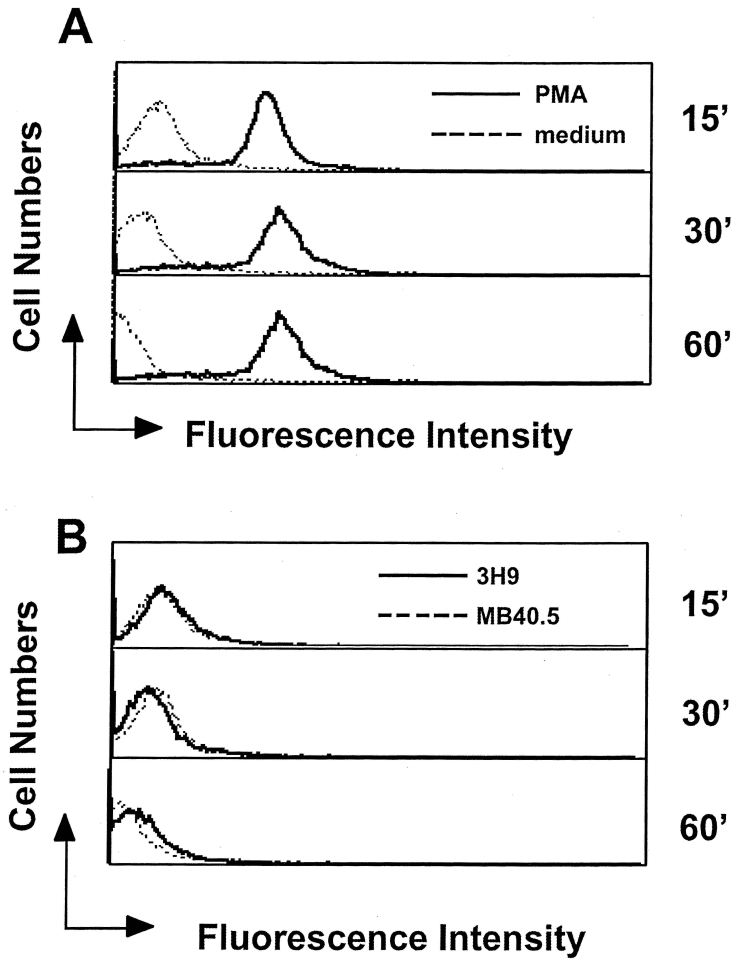

Fig. 6.
Reactive oxygen production on 3H9 reacted monocytes.
Leukocytes were preincubated with 5 μM DCFH-DA at 37 ℃ for 15 min. After preincubation, the mixtures were cultured in the presence (_) or absence (....) of 100 nM PMA for 15, 30 and 60 min at 37 ℃ (A). Preincubated cells were incubated with 10 μg/ml MB40.5 (....) or 10 μg/ml 3H9 (_) for 15, 30 and 60 min at 37 ℃ (B). Gated monocytes were analyzed as described in Material and Methods. The result is a representative of three experiments.

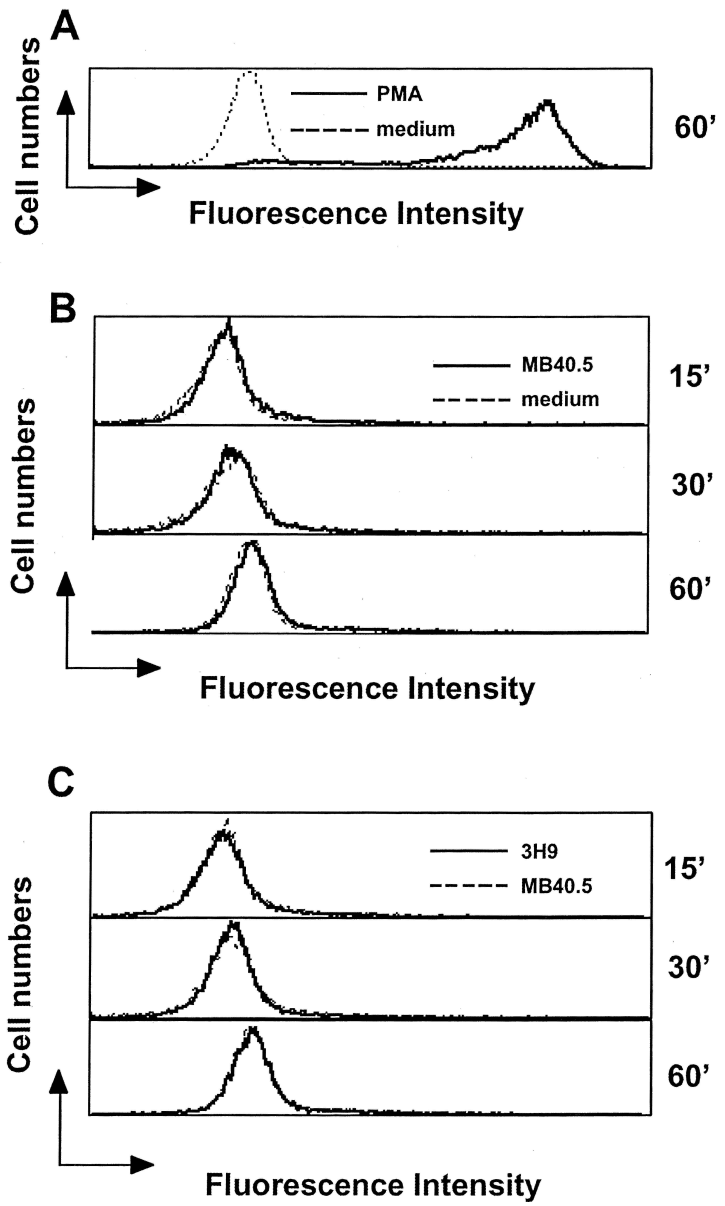

Fig. 7.
FD macropinocytosis of neutrophils.
Leukocytes were incubated for 60 min at 37 ℃ with 1 mg/ml FD in the absence (....) or presence (_) of 100 nM PMA (A). Leukocytes were incubated for 15, 30 and 60 min at 37 ℃ with 1 mg/ml FD. The mixtures were also incubated in the absence (...) or presence (_) of 10 μg/ml MB40.5 (B), or in the presence (....) of 10 μg/ml MB40.5 or 3H9 (_) (C). Neutrophils defined by specific gate were analyzed using FACSCalibur. The result is a representative of four experiments.

treatments did not change the fluorescence intensity on monocytes as same as control mAb (MB40.5) (Fig. 6B). When monocytes were treated with 3H9 and PMA simultaneously, same level of fluorescence intensity shift was observed as PMA stimulation alone (data not shown). These results indicate that the 3H9 treatment could not modulate the reactive oxygen production of monocytes.

No enhancement of neutrophil FD macropinocytosis by treatment with 3H9

Finally, we examined the effect of 3H9 on neutrophil pinocytosis, because our previous data shown that 3H9 modulates several neutrophil functions[1),2),9)]. FD macropinocytosis of neutrophils was dramatically enhanced by PMA (Fig.7A). During incubation, FD macropinocytosis increased gradually, but it was not changed by control mAb treatment. (Fig. 7B). In contrast to the results with monocytes, the 3H9 treatments did not augment neutrophil FD macropinocytosis (Fig. 7C).

DISCUSSION

In this paper, we studies the possible role of GPI-80 in monocyte functions through examining the effect of treatment with a mAb 3H9 to this GPI anchored protein. We first examined the effect of GPI-80 on monocyte endocytosis. In this study we examined phagocytosis, clathrin-dependent pinocytosis, and fluid phase macropinocytosis. Interestingly, macropinocytosis of high MW dextran (MW 70,000) was up-regulated by the 3H9 treatment (Figs.1 and 2), although this treatment was almost no effect on phagocytosis and clathrin-dependent pinocytosis (Figs.4 and 5). Dextran is known to bind the mannose receptor, which is an endocytic receptor and generally highly expressed on human monocytes. On the other hand, macropinocytosis is dependent on membrane ruffling and has been reported to be inhibited by cytochalasin D. Enhancement of FD pinocytosis of monocytes by 3H9 stimulation was completely brocked by cytochalasin D, suggesting that GPI-80 modulates fluid phase macropinocytosis rather than mannose mediated receptor dependent endocytosis. We also investigated the effect of 3H9 on another important monocytes function, reactive oxygen productions, 3H9 had no effect on reactive oxygen production (Fig.6). It is well known that macropinocytosis contributes to the immune response and host defense. A previous report has demonstrated that antigen-presenting cells capture the antigens via macropinocytosis[22)] and that macropinocytosis is also related to endotoxin internalization[23)]. Macropinocytosis by macrophages which is enhanced by M-CSF[24)] and phorbol esters[25)] is a receptor-independent pathway[24),25)]. On the other hand, classical receptor-mediated pinocytosis pathway and phagocytosis by macrophages is induced in a receptor-mediated manner. These findings suggest that GPI-80 may be involved in non-receptor mediated endocytosis but not in receptor mediated ones. Furthermore, GPI-80 may play some physiological roles of monocyte macropinocytosis, such as antigen capture or endotoxin internalization.

We have recently demonstrated that major GPI-80 positive monocytes belong to a CD14 strongly positive ($CD14^{++}$) subpopulation defined by Ziegler-Heitbrock[15),16)]. Almost all (95.7%) monocytes belong to $CD14^{++}$ subpopulation. Although $CD14^{++}$ subpopulation is superior in phagocytosis and reactive oxygen production and inferior in antigen presentation[16)], certain percentages of GPI-80 positive

monocytes bear HLA-DR or HLA-DQ[15]. Together with the results that monocyte macropinocytosis of high MW dextran was up-regulated by anti-GPI mAb 3H9 (Figs. 1 and 2) but not changed monocyte phagocytosis and reactive oxygen production (Figs. 5 and 6), it may be possible that GPI-80 on this GPI-anchored protein-positive monocytes may play some role for antigen processing in $CD14^{++}$ monocytes.

The interesting result that anti-GPI-80 mAb, 3H9 enhanced macropinocytosis of high MW (Figs. 1 and 2) but not low MW molecules (Fig. 3) may be explained as follows. A previous report demonstrated that macromolecules internalized by macrophage endocytosis can be sorted by size[26]. Pinocytozed small solutes can be recycled to extracellular medium more efficiently than large solutes and pinocytozed large solutes can be retained in the cell longer than small solutes[26]. The same group demonstrated that a phosphoinositide 3-kinase inhibitor, wortmannin, inhibits pinocytosis of large solutes more efficiently than that of small solutes[27]. These results suggest that the process of macropinocytosis differs depending on the size of the molecules pinocytozed. Thus, treatment of GPI-80 with 3H9 may enhance macropinocytosis and/or inhibit recycling of large molecules more efficiently than those of small molecules, although the mechanism involved in this phenomenon is still obscure.

The result that neutrophil fluid phase pinocytosis was not enhanced by treatment with mAb to GPI-80 (Fig. 7) was unexpected, since it was enhanced in monocytes. Although the reason for this remains to be clarified, a possibility can be explain as follow. The mechanisms of fluid phase pinocytosis may differ in monocytes/macrophages and neutrophils. Although the effect of cytochalasin B on macrophage pinocytosis may be only partial[28], cytochalasin D significantly inhibits macrophage pinocytosis[29),30]. In contrast, cytochalasin D stimulates neutrophil fluid phase pinocytosis[31]. These results suggest that the mechanisms of pinocytosis of macrophage and neutrophils are somewhat different. From a different view point, the function of GPI-80 may differ in human monocytes and neutrophils. In addition to the discrepancies mentioned above, modulation of human neutrophil adherence by this treatment[1),2),9)] was not detected in human monocytes.

In conclusion, to our knowledge this is the first demonstration that stimulation of a certain cell surface molecules induces the selective enhancement of a portion of monocyte pinocytosis. GPI-80 may play some physiological role in the modulation of monocyte functions. In the future, we will investigate the precise pathway of this enhancement of macropinocytosis.

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