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Potentiation of Chemotactic Peptide-Induced Superoxide Generation by CD38 Ligation in Human Myeloid Cell Lines[1]

Noriko Tsujimoto,* Kenji Kontani,* Shin-ichi Inoue,* Shin-ichi Hoshino,* Osamu Hazeki,* Fabio Malavasi,† and Toshiaki Katada*,[2]

*Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113; and †Institute of Biology and Genetics, University of Ancona Medical School, Ancona, Italy

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CD38 is a type II transmembrane glycoprotein possessing an NAD^+ glycohydrolase activity in its extracellular domain. We previously reported that the ligation of CD38 by a monoclonal antibody (mAb), HB-7, induces the tyrosine phosphorylation of cellular proteins including $p120^{c\text{-}cbl}$ in differentiated human myeloid cell lines and that the phosphorylated $p120^{c\text{-}cbl}$ is capable of binding to phosphatidylinositol (PI) 3-kinase. In the present study, we found that the agonistic anti-CD38 mAb markedly potentiates superoxide generation stimulated by chemotactic *formyl*-Met-Leu-Phe receptors in the CD38-producing cells. HB-7 neither generated superoxide by itself nor enhanced the cell response induced by phorbol 12-myristate acetate, indicating that the potentiating action of the anti-CD38 mAb is specific for the stimulation by the GTP-binding protein (G_i)-coupled membrane receptors. The potentiation by HB-7 was abolished by prior treatment of the cells with a tyrosine kinase inhibitor, pertussis toxin, or a potent PI 3-kinase inhibitor, wortmannin. HB-7 also enhanced the product formation of PI 3-kinase in response to the chemotactic receptor stimulation, without significant changes in the receptor-stimulated accumulations of inositol-1,4,5-trisphosphate, arachidonate release, and intracellular Ca^{2+}. These results indicate that the CD38-induced tyrosine phosphorylation has a cross-talk with the chemotactic receptor/G_i-mediated signal transduction pathway resulting in the enhancement of superoxide generation, probably through the activation of PI 3-kinase.

Key words: CD38, chemotactic receptors, G proteins, tyrosine phosphorylation, superoxide generation.

CD38 is a type II single-transmembrane glycoprotein with the M_r of 46,000 and has no significant homology with other known cell surface proteins (*1*). The analysis of the expression of CD38 is widely used as a phenotypic marker of differentiation or activation of human T and B lymphocytes; the cell surface antigen is expressed at high levels on the cell surface in both the early and activated stages of T and B cell maturation but not in the intermediate ones (*2*). We previously demonstrated that NAD^+ glycohydrolase (NADase) activity induced by all-*trans* RA in human leukemic HL-60 cells is due to the extracellular domain of CD38 (*3*) and that the same molecule is able to bind hyaluronate (*4*). CD38 displayed an amino acid sequence similar to *Aplysia* ADP-ribosyl cyclase, an enzyme catalyzing the formation of cyclic ADP-ribose from NAD^+ (*5*). The cyclic nucleotide has been considered as a new mediator or modulator of Ca^{2+} release from intracellular stores insensitive to inositol-1,4,5-trisphosphate (see Ref. *6* for review). Indeed, CD38 catalyzes not only the hydrolysis of NAD^+ but also the formation and hydrolysis of cyclic ADP-ribose (*7-9*). We recently developed a specific radioimmunoassay for the measurement of cellular cyclic ADP-ribose and showed that the intracellular concentration of cyclic ADP-ribose was correlated with high expression of CD38 in several cell lines (*10*). However, the physiological relevance of the accumulation of cyclic ADP-ribose in the CD38-producing cells remains obscure.

CD38 also appears to be an important immuno-regulatory molecule functioning as a cell-surface receptor, since the binding of anti-CD38 mAbs has been shown to trigger a wide variety of cell functions including cell proliferation, protection from apoptosis, inhibition of cell adhesion, and cytokine production (*11-16*). Thus CD38 ligation by the agonistic mAbs is believed to mimic some of the events triggered by the binding with a natural ligand. In this regard, a cell surface protein (the antigen of Moon-1 mAb) with the M_r of 120,000, which is predominantly produced in endothelial cells, was recently identified as one of the

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[2] To whom correspondence should be addressed. Tel.: +81-3-3812-2111 (Ext. 4750), Fax: +81-3-3815-9604, E-mail: katada@mol.f.u-tokyo.ac.jp

Abbreviations: NADase, NAD^+ glycohydrolase; Bt_2-cAMP, dibutyryl cyclic AMP; fMLP, *N*-formyl-Met-Leu-Phe; G protein, GTP-binding protein; IP_3, inositol-1,4,5-trisphosphate; mAb, monoclonal antibody; PI, phosphatidylinositol; PIP_3, PI-3,4,5-trisphosphate; p85, 85-kDa subunit of PI 3-kinase; pAb, polyclonal antibody; RA, retinoic acid; PY, phosphotyrosine.

CD38-binding proteins (*17*). However, the molecular mechanisms underlying the CD38-mediated signal transduction have not been fully elucidated. Recently, we found that stimulation of CD38 with the agonistic mAbs induces tyrosine phosphorylation of cellular proteins in RA-differentiated HL-60 (*18*) and Bt_2-cAMP-treated THP-1 cells (*19*). One of the prominent tyrosine-phosphorylated proteins was identified as c-*cbl* proto-oncogene product, $p120^{c\text{-}cbl}$. Such protein-tyrosine phosphorylation was also observed in mouse (*20, 21*) and human (*22*) B cells upon CD38 ligation by their mAbs. Interestingly, the epitopes recognized by these agonistic mAbs stimulating protein tyrosine phosphorylation were all mapped on the same carboxyl-terminal sequence of CD38, and the same sequence was also required for its ecto-NADase activity (*23*). More recently, we found that the tyrosine-phosphorylated $p120^{c\text{-}cbl}$ is capable of binding to the p85 regulatory subunit of PI 3-kinase in Bt_2-cAMP-treated THP-1 cells (*24*).

In the present study, we investigated the effect of the agonistic anti-CD38 mAbs on superoxide generation in the CD38-producing human myeloid cells, since our previous results reveal that PI 3-kinase plays an indispensable role in the cell response mediated by the stimulation of G_i-coupled chemotactic fMLP receptors (*25*). We found that the fMLP-induced superoxide generation was markedly enhanced upon CD38 ligation by the agonistic mAb (HB-7). This potentiation appeared to be dependent on the HB-7-induced tyrosine phosphorylation and accompanied by the enhanced accumulation of PI 3-kinase product, PIP_3, in the cells. Our present results indicate that the CD38-induced tyrosine phosphorylation has a cross-talk with the receptor/G_i-mediated signal transduction pathway, resulting in the potentiation of superoxide generation probably through the activation of PI 3-kinase.

MATERIALS AND METHODS

Cell Culture and Differentiation—HL-60 and THP-1 cells were grown in RPMI 1640 containing 10% fetal bovine serum and 200 μg/ml of kanamycin sulfate at 37°C in 95% air and 5% CO_2. HL-60 cells were caused to differentiate either by treatment with 1 μM RA for 24 h followed by subsequent treatment with 0.5 mM Bt_2-cAMP for the next 24 h or by treatment with 0.5 mM Bt_2-cAMP alone for 48 h. THP-1 cells were treated with 0.5 mM Bt_2-cAMP for 3-4 days to produce CD38 (*19*).

Materials—An anti-CD38 mAb, HB-7 (IgG1), was purified from the culture medium of mouse hybridoma HB136 cells as described previously (*18*). Another anti-CD38 mAbs, T16 (IgG1; IOB6) and RFT-10 (IgG2b; #CBL167), were obtained from Cosmo Bio (Tokyo) and Cymbus Bioscience (Southampton, Hants), respectively. The subclass-matched control mouse IgG1 (#64-335) and IgG2b (#64-337) were purchased from ICN Biochemicals (Costa Mesa, CA). The control IgG2a (#MI 10-103) used for an anti-CD38 mAb, IB4, was from Bethyl Laboratories (Montgomery, TX). The anti-CD38 pAb used for immunoblotting was described previously (*19*). The anti-PY mAb, PY-20, and rabbit anti-PY pAb were obtained from Leinco Technologies (Ballwin, MO) and Chemicon International (Temecula, CA), respectively. Sepharose 4B and Protein G-Sepharose 4-FF were from Pharmacia Biotech (Uppsala, Sweden). Superoxide dismutase (bovine erythrocyte) and Fura-2 AM were obtained from Dojindo Laboratories (Kumamoto), herbimycin A was from Wako Pure Chemicals Industries (Osaka), fMLP and cytochrome *c* were from Sigma Chemical (St. Louis, MO). $^{32}P_i$, [^{3}H]arachidonate, and ^{125}I-labeled protein A were from DuPont NEN. The assay system of IP_3 was obtained from Amersham. All other reagents were of analytical grade and from commercial sources.

Superoxide Generation—Superoxide generation was determined by measuring the reduction of cytochrome *c*. The cultured cells were suspended in Hank's solution containing 10 mM Na-HEPES (pH 7.4), 1 mg/ml of BSA, and 1.2 mg/ml of cytochrome *c*. An aliquot (180 μl, 5×10^6 cells/ml) was first incubated at 37°C for 5 min with or without superoxide dismutase (170 units/ml), then further incubated with 20 μl of various concentrations of fMLP and anti-CD38 mAbs (or the control IgGs) at 37°C for the indicated times. The reactions were terminated by adding 10 μl of 40 mM *N*-ethylmaleimide. The reduced cytochrome *c* was determined by spectroscopic analysis at 550 nm. The quantity of reduced cytochrome *c* was calculated with the absorption coefficient of 2.1×10^4 $M^{-1}\cdot cm^{-1}$.

Tyrosine-Phosphorylated Proteins—The analysis of tyrosine-phosphorylated proteins in the cell lysates was described previously (*18*). Briefly, the cells were suspended in serum-free RPMI 1640 containing 10 mM Na-HEPES (pH 7.4) at a density of 2.5×10^7 cells/ml. After preincubation at 37°C for 5 min, the cells (1×10^7 cells/400 μl) were stimulated with various concentrations of anti-CD38 mAbs (or the control IgGs) at 37°C for 2 min. The reaction was terminated by adding the same volume (400 μl) of a lysis buffer consisting of 40 mM Na-HEPES (pH 7.4), 150 mM NaCl, 30 mM NaF, 2 mM Na_3VO_4, 20 mM $Na_4P_2O_7$, 4 mM EDTA, 2% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 4 μg/ml of aprotinin. The cell extracts, after being precleared with Sepharose 4B resin, were immunoprecipitated with PY20 (1 μg) and protein-G Sepharose resin. The resultant immunoprecipitates were subjected to SDS-PAGE (10% of acrylamide), and the separated proteins were transferred onto a polyvinylidene difluoride membrane. The membrane, after being shaken in TBS (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 5% BSA, was incubated with rabbit anti-PY polyclonal antibody at room temperature for 2 h, then further incubated with ^{125}I-labeled protein A in TBS containing 5% BSA. The radioactivity retained on the membrane was visualized with a Fuji BAS 2000 bioimaging analyzer.

PIP_3 Production—PIP_3 production was measured according to the method described by Okada *et al.* (*25*). The differentiated THP-1 cells (4-day culture with Bt_2-cAMP) were suspended at the density of 4×10^7 cells/ml in a labeling medium consisting of 10 mM Na-HEPES (pH 7.4), 136 mM NaCl, 4.9 mM KCl, and 5.5 mM glucose and incubated at 37°C for 30 min with 150 μCi of $^{32}P_i$. The radiolabeled cells were washed twice with the same medium, then resuspended at 5×10^6 cells/ml in the medium containing 1 mM $CaCl_2$. Aliquots (400 μl, 5×10^6 cells/ml) of the suspension were preincubated for 5 min, then incubated with fMLP and anti-CD38 mAbs (or the control IgGs). The reaction was terminated by the addition of 1.55 ml of chloroform/methanol/8% $HClO_4$ (50 : 100 : 5). Then 0.5 ml each of chloroform and 8% $HClO_4$ were added to the mixture, and the lipid phase was separated by

centrifugation. The lipid phase was washed with chloroform-saturated 1 M NaCl containing 1% $HClO_4$ and dried. The extracted lipid was dissolved in 20 μl of chloroform/methanol (95 : 5) and spotted on a TLC plate (Silica gel 60, Merck). Prior to spotting, the plate was once developed with methanol/water (2 : 3) containing 1.2% potassium oxalate and preactivated by heating at 110°C for 20 min. The plate was developed in chloroform/acetone/methanol/acetic acid/water (80 : 30 : 26 : 24 : 14), and radioactivities were visualized with the bioimaging analyzer.

IP_3 Formation—IP_3 formation was determined by use of an assay kit obtained from Amersham. The differentiated THP-1 cells were suspended in Hank's solution containing 10 mM Na-HEPES (pH 7.4), 1 mM $CaCl_2$, and 1 mg/ml of BSA. Aliquots (200 μl, 2×10^7 cells/ml) of the suspension were preincubated at 37°C for 5 min, then incubated with fMLP and anti-CD38 mAbs (or the control IgGs) at 37°C for the indicated times. The reaction was terminated by adding 200 μl of 15% trichloroacetic acid, and proteins were precipitated by centrifugation. The supernatant was extracted four times with 3 ml of water-saturated diethyl ether to remove trichloroacetic acid. IP_3 content in the samples was measured by the procedure described in the assay protocol.

Arachidonate Release—The differentiated THP-1 cells were incubated with [^{3}H]arachidonate (0.1 μCi/ml) at 37°C for 4 h in the culture medium. The radiolabeled cells were washed twice with and suspended in Hank's solution containing 10 mM Na-HEPES (pH 7.4), 1 mM $CaCl_2$, and 1 mg/ml of BSA. Aliquots (200 μl, 1×10^7 cells/ml) of the suspension were preincubated for 5 min, then incubated with fMLP and anti-CD38 mAbs (or the control IgGs). The reaction was terminated by adding 200 μl of phosphate-buffered saline (Ca^{2+} and Mg^{2+} free) containing 2 mM EGTA. The reaction mixture was centrifuged, and radioactivity in the supernatant was measured.

Intracellular Ca^{2+} Concentration—Intracellular Ca^{2+} concentration was determined by measuring fluorescence intensity of Fura-2. The cultured THP-1 cells were washed twice with and suspended in Hank's solution containing 10 mM Na-HEPES (pH 7.4), 1 mM $CaCl_2$, and 1 mg/ml of BSA. The cells were loaded with 4 μM Fura-2 AM and incubated at 37°C for 30 min. The Fura-2-loaded cells were washed again with and resuspended in the same buffer. Aliquots of the suspension (500 μl, 1.5×10^7 cells/ml) were pipetted into a cuvette and incubated with fMLP and anti-CD38 mAbs (or the control IgGs). The fluorescence intensity of Fura-2 was measured using a HITACHI F-2000 spectrophotometer at excitation wavelengths of 340 and 380 nm with an emission wavelength of 510 nm.

Miscellaneous—The cyclic ADP-ribose content of the cells was measured by a specific radioimmunoassay described previously (*10*). The immunoblot procedure with anti-CD38 pAb was described previously (*19*). All experiments were repeated at least twice with different batches of cultured cells. Hence, most of the data shown are averages of duplicate or triplicate determinations of a single batch of cells which varied within less than 10%.

RESULTS

Potentiation by Anti-CD38 mAb of Chemotactic Receptor-Mediated Superoxide Generation in Human Myeloid Cell Lines—HL-60 cells were first treated with RA to produce CD38 (*3*) and analyzed for superoxide generation in response to fMLP. The chemotactic peptide, however, had little effect on superoxide generation in the RA-treated HL-60 cells (data not shown), probably due to no induction of fMLP receptors (*26*). Thus, the CD38-producing cells were further treated with Bt_2-cAMP to acquire the sensitivity to fMLP. As shown in Fig. 1A, fMLP stimulated superoxide generation in the RA *plus* Bt_2-cAMP-treated cells. The addition of an anti-CD38 mAb, HB-7, or the control IgG1 alone had no stimulatory effect on superoxide generation. Nevertheless, HB-7 markedly potentiated the fMLP-induced superoxide generation. The specific action of HB-7 on CD38 was confirmed by its inability to potentiate the fMLP-induced cell response in HL-60 cells which had been treated with Bt_2-cAMP alone to differentiate without the production of CD38 (Fig. 1B). Recently, we found that CD38 is also induced in human monocytic leukemia THP-1 cells after their treatment with Bt_2-cAMP (*19*). The Bt_2-cAMP-treated THP-1 cells generated superoxide in response to fMLP, and HB-7 markedly enhanced the fMLP-induced superoxide generation (Fig. 1C). In the following series of experiments, we used the Bt_2-cAMP-treated THP-1 cells, since this CD38-producing cell line responded well to fMLP to generate superoxide.

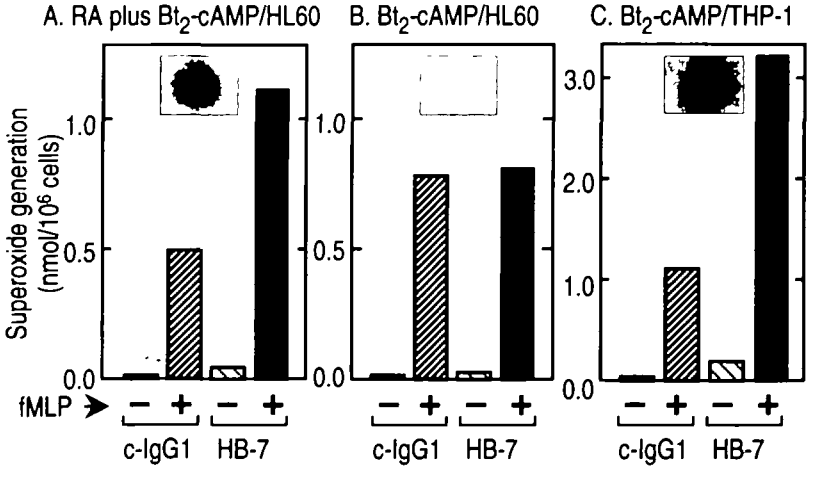

Fig. 1. **Effect of an anti-CD38 mAb (HB-7) on fMLP-induced superoxide generation in differentiated human myeloid cells.** HL-60 cells were first cultured with RA and then Bt_2-cAMP (A, RA plus Bt_2-cAMP/HL-60) or with Bt_2-cAMP alone (B, Bt_2-cAMP/HL-60). THP-1 cells were also treated with Bt_2-cAMP (C, Bt_2-cAMP/THP-1) as described in "MATERIALS AND METHODS." These cells were incubated with (+) or without (−) 1 μM fMLP at 37°C for 10 min in Hank's buffer containing 5 μg/ml of the subtype-matched control IgG1 (c-IgG1) or anti-CD38 mAb, HB-7. Superoxide generation was measured as described in "MATERIALS AND METHODS" and is expressed as nmol of cytochrome *c* reduction per 10^6 cells. Insets: Cell lysates were prepared from the cultured cells and immunoprecipitated with HB-7. The precipitated samples were separated by SDS-PAGE and subjected to immunoblotting with anti-CD38 pAb.

Figure 2A shows the time courses of fMLP-induced superoxide generation in the presence and absence of HB-7. The initial rate as well as the maximum extent of the fMLP-induced superoxide generation were enhanced by the presence of HB-7. The potentiating action of HB-7 was further investigated by pretreatment of the cells with the agonistic mAb before the addition of fMLP (Fig. 2B). Pretreatment for up to 5 min did not further enhance, and longer pretreatment rather diminished, the potentiating action of HB-7, indicating that an optimal effect of the agonistic mAb was very rapidly produced after its addition to the cells.

Figure 3A shows the effect of HB-7 on various concentrations of fMLP. The anti-CD38 mAb potentiated the fMLP-induced superoxide generation without changing the EC_{50} of fMLP. HB-7 enhanced superoxide generation induced by another chemoattractant, C5a (data not shown), of which receptors are also coupled to heterotrimeric G_i. On the contrary, HB-7 failed to enhance superoxide generation stimulated by phorbol 12-myristate acetate (Fig. 3B), which bypassed the receptors/G_i to stimulate the cells. Thus, the potentiating action of HB-7 was specifically observed for the stimulation by G_i-coupled membrane receptors.

Potentiation of Superoxide Generation Dependent on Anti-CD38 mAb-Induced Tyrosine Phosphorylation—In previous papers (*18, 19*), we showed that ligation of CD38 with HB-7 rapidly induces tyrosine phosphorylation of cellular proteins in RA-differentiated HL-60 and Bt_2-cAMP-treated THP-1 cells. We also reported that the epitope of HB-7 is identical to those of other agonistic anti-CD38 mAbs, T16 and IB4, which trigger various cell functions in human T and B cell lines (*23*). However, the abilities of the three agonistic mAbs to stimulate the tyrosine phosphorylation were different from one another. Thus, we investigated the effects of various anti-CD38 mAbs on the fMLP-induced superoxide generation in Bt_2-cAMP-treated THP-1 cells. As shown in Fig. 4, T16 potentiated fMLP-induced superoxide generation to the same extent as did HB-7. However, IB4 had no potentiating action on the superoxide generation. The different potency observed with the three CD38 mAbs was well correlated with our previous findings that the ability of IB4 to stimulate protein-tyrosine phosphorylation was much lower than those of HB-7 and T16 in CD38-producing THP-1 cells (*23*). As expected, another anti-CD38 mAb, RFT-10, which failed to stimulate tyrosine phosphorylation (Fig. 5A), had no potentiating action on the fMLP-induced superoxide generation (Fig. 5B).

The relationship between the potentiation of superoxide generation and the protein tyrosine phosphorylation was further investigated with different concentrations of HB-7. As shown in Fig. 5D, the half-maximum effect of HB-7 on the fMLP-induced superoxide generation was observed at a concentration of several μg/ml. HB-7-induced tyrosine phosphorylation was also dependent on its concentration (Fig. 5C), and the concentration-dependency for the overall phosphorylation pattern appeared to be similar to that observed for the potentiating action of HB-7 on fMLP-induced superoxide generation. The effect of a tyrosine kinase inhibitor, herbimycin A, was also investigated. Not only superoxide generation stimulated by fMLP *plus* HB-7 but

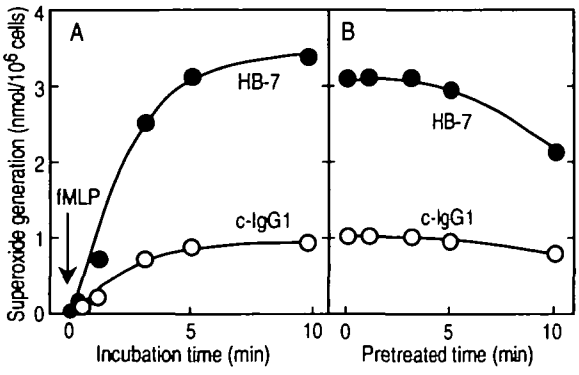

Fig. 2. **Potentiation of fMLP-induced superoxide generation by the anti-CD38 mAb in Bt_2-cAMP-treated THP-1 cells.** A: The Bt_2-cAMP-treated THP-1 cells were incubated with 1 μM fMLP at 37°C for the indicated times in Hank's buffer containing 5 μg/ml of the control IgG1 (○) or HB-7 (●) for the measurement of superoxide generation. B: The Bt_2-cAMP-treated THP-1 cells were first pretreated with 5 μg/ml of control IgG1 (○) or HB-7 (●) for the indicated times, then further incubated with 1 μM fMLP for 10 min. Superoxide generation stimulated by fMLP in the second 10-min incubation was measured as described in "MATERIALS AND METHODS."

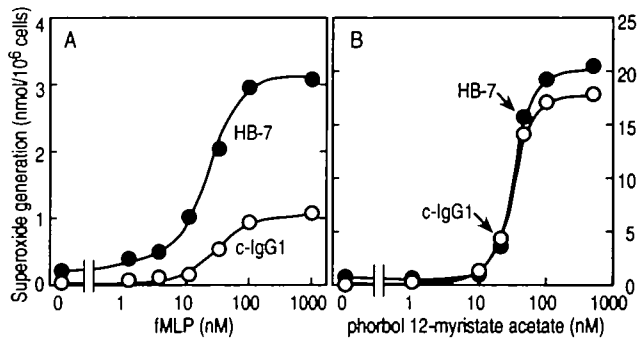

Fig. 3. **Potentiation by the anti-CD38 mAb specifically observed for the G_i-coupled receptor-mediated stimulation of superoxide generation.** The Bt_2-cAMP-treated THP-1 cells were incubated at 37°C for 10 min with the indicated concentrations of fMLP (A) or phorbol 12-myristate acetate (B), in Hank's buffer containing 5 μg/ml of the control IgG1 (○) or HB-7 (●) for the measurement of superoxide generation.

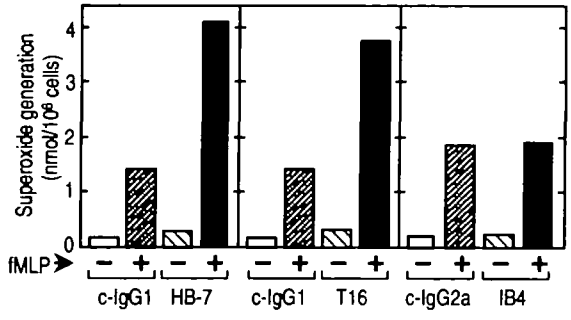

Fig. 4. **Effects of various anti-CD38 mAbs on fMLP-induced superoxide generation.** The Bt_2-cAMP-treated THP-1 cells were incubated at 37°C for 10 min with (+) or without (−) 1 μM fMLP in the presence of 5 μg/ml of the subtype-matched control IgGs (c-IgG1 or c-IgG2a) or anti-CD38 mAbs (HB-7, T16, or IB4) for the measurement of superoxide generation.

also the HB-7-induced tyrosine phosphorylation was progressively inhibited as the concentration of the kinase inhibitor was increased (Fig. 6). Superoxide generation stimulated by phorbol 12-myristate acetate was, however, not altered in the herbimycin A-treated cells (data not shown). Thus, the tyrosine phosphorylation of cellular proteins appeared to be responsible for the potentiating action of the agonistic anti-CD38 mAb.

Possible Involvement of PI 3-Kinase Activation in the Potentiating Action of Agonistic Anti-CD38 mAb—We have reported that PI 3-kinase plays an important role in the G_i-coupled fMLP receptor-induced stimulation of superoxide generation (*25, 27*) and that the tyrosine-phosphorylated $p120^{c\text{-}cbl}$ is capable of binding to the p85 regulatory subunit of PI 3-kinase in Bt_2-cAMP-treated THP-1 cells (*24*). As shown in Fig. 7, the fMLP-induced superoxide generation, together with its potentiation by HB-7 (panel A), was almost completely abolished not only by the addition of a specific PI 3-kinase inhibitor, wortmannin (panel B), but also by the prior treatment of the cells with pertussis toxin (panel C). These results indicate that the activations of G_i and PI 3-kinase are indeed involved in the signal transduction pathway leading to fMLP-induced superoxide generation and its potentiation by HB-7.

We finally investigated the effects of HB-7 on various intracellular messengers which may be responsible for fMLP-induced superoxide generation, and the results are summarized in Fig. 8. It has been reported that there are increases of PIP_3 formation, IP_3 formation, arachidonate release, and intracellular Ca^{2+} elevation upon stimulation of cells with fMLP (see Refs. *28* and *29* for review). Such were also observed for Bt_2-cAMP-treated THP-1 cells. As shown in Fig. 8A, PIP_3 formation stimulated by fMLP was significantly enhanced by the presence of HB-7, though the mAb alone had no stimulatory effect. In contrast, HB-7 did not influence the fMLP-induced IP_3 formation (Fig. 8B), arachidonate release (Fig. 8C) or intracellular Ca^{2+} elevation (Fig. 8D). A slow and slight elevation of intracellular Ca^{2+} was only noted in the THP-1 cells incubated with HB-7

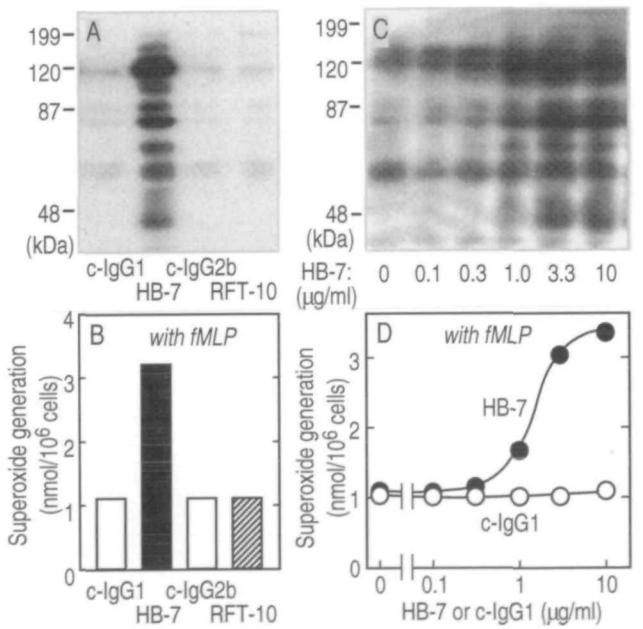

Fig. 5. **Potentiation of fMLP-induced superoxide generation dependent on anti-CD38 mAb-induced protein-tyrosine phosphorylation.** A: The Bt_2-cAMP-treated THP-1 cells were incubated at 37°C for 2 min with 5 μg/ml of the subtype-matched control IgGs (c-IgG1 or c-IgG2b) or anti-CD38 mAbs (HB-7 or RFT-10). Tyrosine-phosphorylated proteins in the cell lysate were immunoprecipitated with PY20, separated by SDS-PAGE, then visualized as described in "MATERIALS AND METHODS." The molecular weight markers used were obtained from Bio-Rad and are indicated in kilodaltons (kDa). B: The THP-1 cells were incubated with 1 μM fMLP at 37°C for 10 min in the presence of 5 μg/ml of the control IgGs or anti-CD38 mAbs for the measurement of superoxide generation. C: The THP-1 cells were incubated at 37°C for 2 min with the indicated concentrations of HB-7 in the presence of 1 μM fMLP for the measurement of tyrosine-phosphorylated proteins. D: The THP-1 cells were incubated with 1 μM fMLP at 37°C for 10 min in the presence of the indicated concentrations of the control IgG1 (○) or HB-7 (●) for the measurement of superoxide generation.

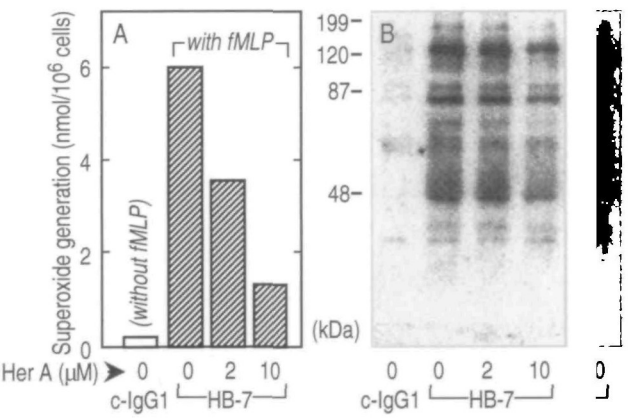

Fig. 6. **The anti-CD38 mAb-induced potentiation of superoxide generation and protein-tyrosine phosphorylation as affected by a tyrosine-kinase inhibitor.** The Bt_2-cAMP-treated THP-1 cells were first cultured for 5 h with the indicated concentrations of hyrbimycin A (Her A). A: The cultured cells were further incubated with or without 1 μM fMLP at 37°C for 10 min in the presence of 5 μg/ml of the control IgG1 or HB-7 for the measurement of superoxide generation. B: The cultured cells were incubated at 37°C for 2 min in the presence of 5 μg/ml of the control IgG1 or HB-7 for the measurement of tyrosine-phosphorylated proteins.

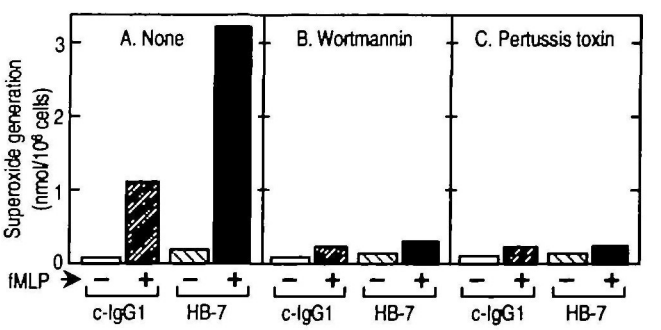

Fig. 7. **Inhibition by a PI 3-kinase inhibitor or pertussis toxin of fMLP-induced superoxide generation and its potentiation by the anti-CD38 mAb.** The Bt_2-cAMP-treated THP-1 cells were first treated without (A) or with 1 μM wortmannin for 10 min (B) or 50 ng/ml of pertussis toxin for 4 h (C). The treated cells were then incubated with (+) or without (−) 1 μM fMLP at 37°C for 10 min in the presence of 5 μg/ml of control IgG1 or HB-7 for the measurement of superoxide generation.

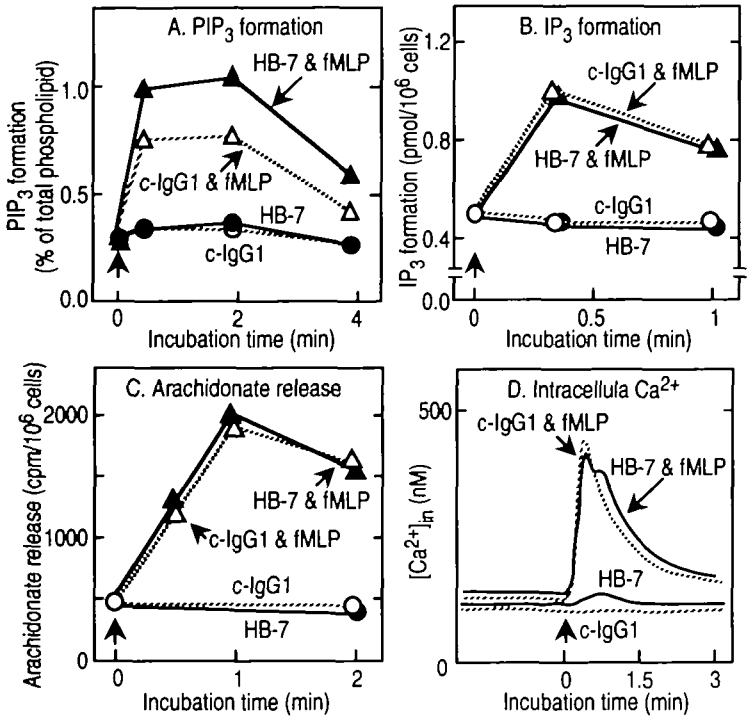

Fig. 8. **Effects of the anti-CD38 mAb on fMLP-induced increases of intracellular messengers.** The Bt_2-cAMP-treated THP-1 cells were incubated with (triangles) or without (circles) 1 μM fMLP in the presence of 5 μg/ml of the control IgG1 (open symbols) or HB-7 (closed symbols). After incubation at 37°C for the indicated times, PIP_3 formation (A), IP_3 formation (B), arachidonate release (C), and intracellular Ca^{2+} elevation (D) were measured as described in "MATERIALS AND METHODS."

alone (Fig. 8D). We also measured cyclic ADP-ribose contents in the cells, since CD38 catalyzes not only the hydrolysis of NAD^+ to ADP-ribose but also the formation of cyclic ADP-ribose (*7-9*). In accordance with our previous report (*10*), the CD38-producing THP-1 cells had a high content of cyclic ADP-ribose: 51.4 ± 3.6 pmol/10^8 cells under the non-stimulated conditions. There were, however, no significant differences in the cyclic nucleotide contents among fMLP and/or HB-7-treated cells (data not shown). Thus, the enhancement of PIP_3 formation appeared to be responsible for the potentiating action of HB-7 on fMLP-induced superoxide generation.

DISCUSSION

In the present study, cells of two human myeloid cell lines, HL-60 and THP-1, were treated with RA *plus* Bt_2-cAMP and Bt_2-cAMP alone, respectively, to produce CD38 (*3, 19*). Both types of differentiated cells also contained fMLP receptors, which enabled us to investigate the effect of anti-CD38 mAbs on the chemotactic receptor-mediated superoxide generation. Thus, we could observe the stimulatory effect of an agonistic anti-CD38 mAb, HB-7, on superoxide generation, which is one of physiologically important cell functions mediated through G_i-coupled receptors in these cell types (Figs. 1 and 2). HL-60 cells treated with Bt_2-cAMP alone did not produce CD38 and failed to response to HB-7, indicating that the potentiation of superoxide generation depends on the cell-surface induction of CD38.

HB-7 was unable to generate superoxide without the G_i-coupled receptor stimulation and did not potentiate the cell response induced by phorbol 12-myristate acetate, which bypasses the receptor-mediated signaling (Fig. 3). The potentiation of superoxide generation was also observed with another anti-CD38 mAb (Fig. 4), and the potentiating action appeared to be dependent on the agonistic mAb-induced tyrosine phosphorylation of cellular proteins (Figs. 5 and 6). In addition, both the fMLP-induced superoxide generation and its potentiation by HB-7 were almost completely abolished by prior treatment of the cells with pertussis toxin (Fig. 7). These results indicate that CD38-induced protein-tyrosine phosphorylation communicates with the fMLP receptor/G_i-mediated signal transduction pathway leading to superoxide generation.

To investigate the molecule(s) responsible for the potentiation of superoxide generation, we evaluated various intracellular messengers which are known to play key roles in the fMLP-induced signaling (Fig. 8). We observed the elevation of intracellular Ca^{2+} concentration, IP_3 formation, and arachidonate release upon stimulation of the differentiated THP-1 cells with fMLP. However, HB-7 did not influence any of these intracellular messengers. In contrast, fMLP-induced PIP_3 formation was significantly enhanced by the presence of HB-7, suggesting that PI 3-kinase activation is stimulated by the CD38 ligation. In this regard, we previously reported an indispensable role of PI 3-kinase in the fMLP-mediated superoxide generation in guinea pig granulocytes using a specific inhibitor of the lipid kinase, wortmannin (*25*). The complete abolishment by wortmannin of the present superoxide generation supports this idea (Fig. 7).

Several intracellular proteins were tyrosine-phosphorylated upon CD38 ligation by HB-7 in Bt_2-cAMP-treated THP-1 cells (Ref. *19*, also see Fig. 5A), and one of the prominent proteins has been identified as $p120^{c\text{-}cbl}$ (*18*). Moreover, the tyrosine-phosphorylated $p120^{c\text{-}cbl}$ was capable of binding to the p85 regulatory subunit of PI 3-kinase (*24*). Such association between tyrosine-phosphorylated $p120^{c\text{-}cbl}$ and PI 3-kinase was also reported in human

immature B cell lines upon CD38 ligation by another anti-CD38 mAb, T16 (*30*), whose epitope we have identified as being the same as that of HB-7 (*23*). These findings, together with the present results, suggest that the associated form of PI 3-kinase with $p120^{c\text{-}cbl}$ might be responsible for the potentiation of fMLP-induced superoxide generation, though there is no direct evidence showing that this form is a primed, higher-sensitive PI 3-kinase for the G_i-mediated activation.

At present, we cannot rule out the possibility that tyrosine-phosphorylated protein(s) other than $p120^{c\text{-}cbl}$ is involved in the potentiating action of the agonistic anti-CD38 mAb. In relation to this, we previously reported that the fMLP receptor-induced formation of PIP_3 is synergistically increased by the simultaneous addition of insulin, which causes the tyrosine phosphorylation of insulin receptor substrate-1, leading to the association with and the activation of PI 3-kinase, in the same Bt_2-cAMP-treated THP-1 cells (*27*). Alternatively, it is also likely that the HB-7-induced tyrosine phosphorylation might increase PIP_2, the substrate of PI 3-kinase, or decrease the breakdown of PIP_3. The molecular mechanism underlying the marked stimulation by HB-7 of fMLP-induced superoxide generation has not been fully resolved in the present study. Nevertheless, our results clearly indicate that the CD38-induced tyrosine phosphorylation has a cross-talk with the receptor/G_i-mediated signal transduction pathway. This cross-talk appeared as an increase in the formation of PIP_3, an important intracellular messenger for the cell signaling.

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