Volume 273, number 1,2, 55–58 FEBS 09005 October 1990

Signal transduction in human monocytes and granulocytes through the PI-linked antigen CD14

Fridtjof Lund-Johansen[1], Johanna Olweus[1], Ånen Aarli[2] and Robert Bjerknes[3]

[1]*Department of Pathology, The Gade Institute,* [2]*The Broegelmann Research Laboratory for Microbiology* and [3]*Department of Pediatrics, University of Bergen, Haukeland Hospital, N-5021 Bergen, Norway*

Received 20 August 1990

A possible role for the PI-linked CD14 molecule in human monocyte and granulocyte signal mediation was investigated. Using flow cytometry and the fluorescent indicators Fluo-3 and dihydrorhodamine-123 it was shown that crosslinking of the CD14 molecule induces an increase in monocyte and granulocyte cytoplasmic calcium concentration and monocyte H_2O_2 production. These responses were found to be independent of IgG Fc receptors and suggest an intrinsic signal mediating capacity of the CD14 molecule.

CD14; Signal transduction; Calcium; Oxidative metabolism; Leukocyte; Flow cytometry.

1. INTRODUCTION

Che CD14 antigen is a 55 kDa glycoprotein present on the membrane of bone marrow and peripheral blood monocytic cells, macrophages and peripheral blood granulocytes [1,2]. The molecule is linked to the leukocyte membrane by phosphatidyl inositol (PI) [3], and has been found in soluble form in human plasma and urine [4].

A possible signal transducing capacity of CD14 has been speculated on, but earlier studies have not been able to demonstrate intrinsic signal mediating properties of the antigen [5], and monocyte activation by IgG anti-CD14 mAbs were reported to be the result of IgG Fc receptor interaction [5]. During the 4th Workshop of Leukocyte Differentiation Antigens it was demonstrated that some mAbs to CD14 significantly increased monocyte chemiluminescence during phagocytosis [6]. This was seen with both IgG and IgM mAbs, suggesting the possibility of an IgG Fc receptor independent stimulation of monocytes. Even though there was significant heterogeneity of effects within the CD14 mAb panel, these results may indicate a role for CD14 in monocyte function.

Recently, several studies have shown that crosslinking of certain surface antigens by mAbs can induce leukocyte activation [7–9]. Extensive crosslinking may, however, be necessary, and even when IgM antibodies are applied, a response is usually only seen following the addition of a secondary antibody [7,8]. Heterogeneity in calcium mobilizing effects of different mAbs to the same antigen has also been reported. Thus, insufficient crosslinking of the CD14 antigen, a varying capacity of different mAbs to crosslink the antigen and variable IgG Fc receptor interaction could explain the inconclusive results from earlier studies on CD14 function [2,5,6]. In order to reinvestigate the postulate of CD14 having a role in signal transduction, we studied changes in leukocyte calcium concentration and H_2O_2 production following antigen crosslinking with a panel of CD14 mAbs including the IgM mAb Mo2 and F(ab')$_2$ fragments of an IgG CD14 mAb. As secondary antibodies subtype specific goat anti mouse immunoglobulins were applied.

Correspondence address: F. Lund-Johansen, Department of Pathology, The Gade Institute, University of Bergen, Haukeland Hospital, N-5021 Bergen, Norway

2. MATERIALS AND METHODS

2.1. *Leukocytes*

Venous blood from 3 normal volunteers was drawn into EDTA vacutainers and the red cells were lysed in a solution of 0.8% NH_4Cl, 0.08% $NaHCO_3$ and 0.08% EDTA (all from Sigma Chemicals, St. Louis, MO, USA). The leukocytes were then washed twice in PBS without calcium and magnesium containing 0.5% bovine serum albumine (PBS-BSA).

2.2. *Monoclonal antibodies (mAbs)*

Mo2 (CD14, IgM) (from tissue culture supernatant) was provided by Meryl Foreman, Coulter Immunology (Florida, USA). MEM15, MEM18 (both CD14, IgG_1) and MEM91 (CDw17, IgM) were generously provided by Dr Vaclav Horejsi (Prague, Czechoslovakia). VIM2 (CDw65, IgM) was the kind gift of Dr Walter Knapp (Vienna, Austria). DAKO M1 (CD15, IgM, tissue culture supernatant) was supplied by Marianne Kjærvig Broe, DAKO Patts a/s (Copenhagen, Denmark). AML-2-23 (CD14, IgG2b) was purchased from Meda Rex (West Lebanon, NH, USA). F(ab')$_2$ fragments of MEM15 were prepared by pepsin digestion and purified by Protein A chromatography. The preparation was found to be free of whole IgG in SDS-PAGE analysis.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2.3. *Polyclonal secondary antibodies*

$F(ab')_2$ fragments of human adsorbed goat anti-mouse IgM (μ-chain specific) GAM-M and goat anti-mouse IgG heavy and light chains (GAM-HL) were supplied by Jackson Immunoresearch (West Grove, PA, USA) and DAKO-Patts (Copenhagen, Denmark). The purity of these reagents was controlled by HPLC.

2.4. *Measurement of fluctuations in intracellular calcium concentration*

Leukocyte intracellular calcium changes were monitored by using the green fluorescent calcium indicator Fluo-3 (Molecular Probes, Eugene, OR, USA) [10]. Leukocytes (10^7/ml) were incubated in PBS-BSA containing 1 μM Fluo-3-acetoxymethylester, washed and labeled with mAbs for 20 min at room temperature. The samples were then washed once and resuspended in Hepes buffered saline containing 0.5% BSA and 5 mM glucose (pH 7.3). Prior to flow cytometric analysis, 100 μl of the cell suspension was mixed with 100 μl Hanks balanced salt solution with 0.5% BSA (HBSS-BSA).

Lymphocytes, monocytes and granulocytes were discriminated by flow cytometric measurements of cellular light scatter (see figures). Basal cytoplasmic calcium level was then determined for each leukocyte subset by gated analysis and recorded for about 30 s before GAM-M or GAM-HL (5–30 μg) was added. Fluctuations in cytoplasmic free calcium concentration were recognized as alterations in Fluo-3 fluorescence intensity over time. Fluo-3 fluorescence is dependent of cellular dye concentration as well as cytoplasmic calcium concentration [10]. No attempts were therefore made to quantify absolute concentrations of intracellular calcium.

2.5. *Measurement of leukocyte H_2O_2 production*

Leukocytes were stained with saturating concentrations of Mo2, washed and resuspended in HBSS-BSA containing 15 μg/ml dihydrorhodamine-123 (DHR-123) (Molecular Probes, Oregon, USA) [11]. GAM-M (15 μg) or PBS was then added and the samples incubated at 37°C for 25 min. The cells were washed twice and kept on ice until flow cytometric measurement.

2.6. *Flow cytometry*

Flow cytometric measurements were performed with a Coulter Epics V (calcium measurements) or with a Coulter Profile II (both from Coulter Electronics, Luton, UK).

All experiments were performed in triplicate with four parallel samples in each experiment.

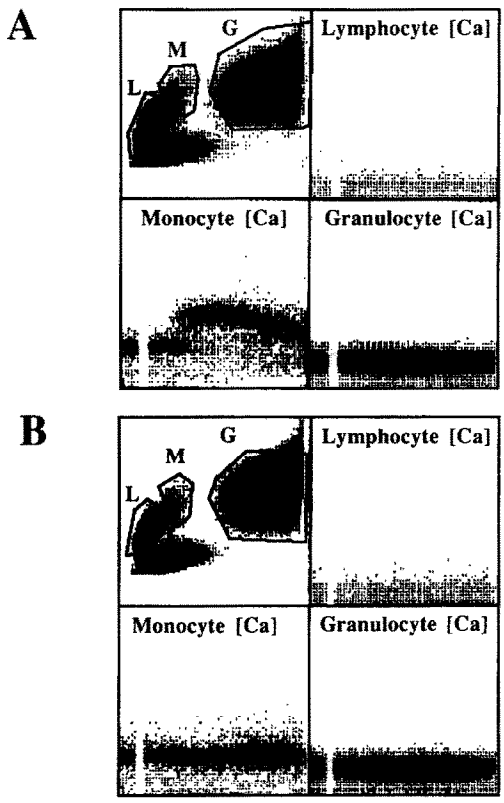

Fig. 1. (A) Flow cytometric determination of fluctuations of leukocyte intracellular calcium concentrations following crosslinking of CD14 using an IgM anti-CD14 mAb. Upper left quadrant: leukocyte discrimination by light scatter (L = lymphocytes; M = monocytes; and G = granulocytes) (*x*-axis: 90° light scatter; and *y*-axis; 4° light scatter). Upper right, lower left and lower right quadrants: Fluo-3 fluorescence (*y*-axis; log scale) gated from lymphocytes, monocytes and granulocytes, respectively, and displayed versus time (*x*-axis; linear scale). The basal level of intracellular calcium was determined for about 30 s. 15 μg GAM-M was then added (interruption of curve), and the measurement continued until 256 s. (B) Flow cytometric determination of leukocyte intracellular calcium concentrations following addition of 30 μg GAM-M to leukocytes preincubated with VIM2 (CDw65, IgM).

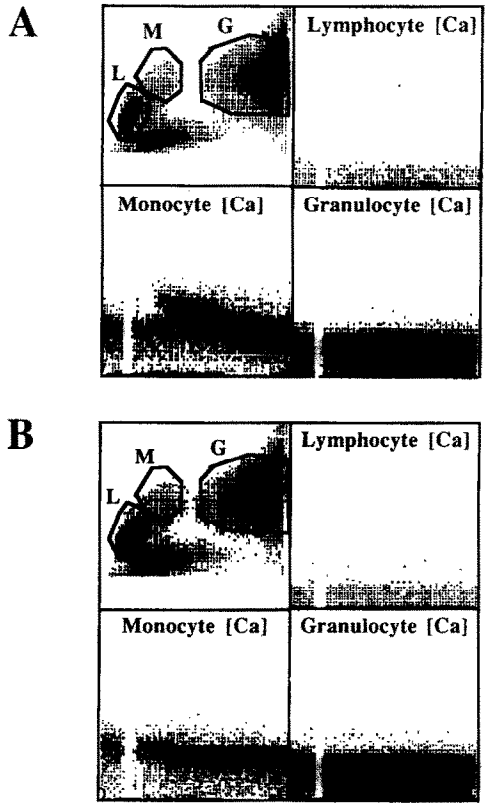

Fig. 2. (A) Flow cytometric determination of fluctuations of leukocyte intracellular calcium concentrations following crosslinking of CD14 using an IgG_1 anti-CD14 mAb. Upper left quadrant: leukocyte discrimination by light scatter (L = lymphocytes; M = monocytes; and G = granulocytes) (*x*-axis: 90° light scatter; and *y*-axis: 4° light scatter). Upper right, lower left and lower right quadrants: Fluo-3 fluorescence (*y*-axis; log scale) gated from lymphocytes, monocytes and granulocytes, respectively, and displayed versus time (*x*-axis; linear scale). The basal level of intracellular calcium was determined for about 30 s. Ten μg of GAM-HL was then added (interruption of curve), and the measurement continued until 256 s. (B) Flow cytometric determination of leukocyte intracellular calcium concentrations following addition of 30 μg GAM-M to leukocytes preincubated with MEM 18 (CD14, IgG_1).

3. RESULTS

3.1. *Cytoplasmic calcium fluxes*

Following addition of GAM-M to cells preincubated with Mo2 (CD14), a rapid increase in monocyte cytoplasmic calcium concentration was observed (Fig. 1A). The rise was followed by a slow decrease. No other cells responded to the stimulus (Fig. 1A), and no effect was demonstrated by Mo2 or GAM-M alone (not shown).

A rise in monocyte calcium concentration was also demonstrated by addition of GAM-HL to leukocytes preincubated with the IgG_1 antibody MEM18 (CD14) (Fig. 2A). No response, however, was observed when GAM-M was used as the secondary antibody, even when used in higher concentrations than those used for Mo2 (Fig. 2B). Similar results were obtained with two other CD14 mAbs, MEM15 and AML-2-23. These mAbs did also induce a calcium flux in granulocytes (not shown).

An increase in monocyte cytoplasmic calcium was also seen when GAM-HL was added to cells preincubated with $F(ab')_2$ fragments of MEM15 (Fig. 3). In addition, a subset of granulocytes (about 40%) responded (Fig. 3).

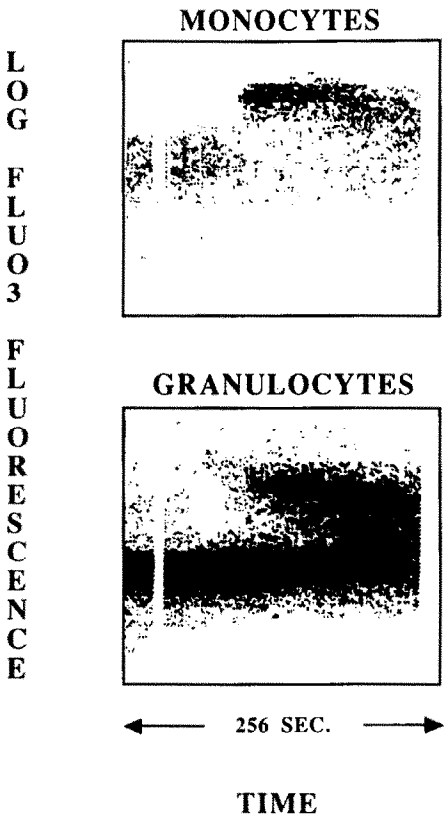

Fig. 3. Flow cytometric determination of intracellular calcium fluxes in monocytes and granulocytes following crosslinking of CD14 with $F(ab')_2$ fragments of MEM15. Monocyte and granulocyte Fluo-3 fluorescence (log scale, *y*-axis) are displayed versus time (*x*-axis, lin scale). Leukocyte subsets were recognized as described in Fig. 1.

Two other IgM antibodies binding to monocyte antigens, VIM2 (CDw65) (Fig. 1B), MEM91 (CDw17) (not shown), and one binding to granulocytes, DAKO M1 (CD15) (not shown), did not affect the calcium concentration of any leukocyte population under the same experimental conditions.

3.2. *Production of H_2O_2*

Monocytes that had been incubated with both Mo2 and GAM-M showed an increase in rhodamine-123 fluorescence of 268 ± 33% (mean ± SE; $n = 3$) as compared to samples incubated with Mo2 or GAM-M alone (Fig. 4). Granulocyte rhodamine-123 fluorescence increased only by 30 ± 12% (Fig. 4).

4. DISCUSSION

We have demonstrated an increase in monocyte and granulocyte cytoplasmic calcium concentration and monocyte H_2O_2 production following crosslinking of the CD14 antigen. The pattern and specificity of the responses suggest a role for CD14 in transmembrane signal mediation. Several lines of evidence suggest that

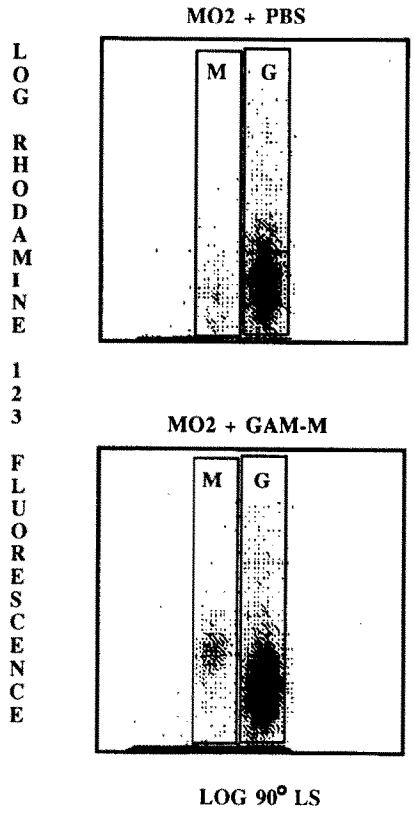

Fig. 4. Flow cytometric measurement of leukocyte H_2O_2 production following CD14 crosslinking. Monocyte and granulocyte rhodamine-123 fluorescence (log scale, *y*-axis) are displayed versus 90° light scatter (90°LS) (log scale, *x*-axis). The upper diagram illustrates cells incubated only with Mo2, whereas the lower shows cellular rhodamine-123 fluorescence following crosslinking of Mo2 by GAM-M. M = monocytes; G = granulocytes.

these responses are independent of cellular IgG Fc receptors. First, a positive response was seen when crosslinking was performed with a purified IgM primary antibody and F(ab')$_2$ fragments of the secondary antibody that had been adsorbed to human immunoglobulins. Second, the secondary antibody was shown to be highly specific for IgM, as no calcium flux was observed when added to cells labeled with IgG antibodies to CD14 (MEM15, MEM18 or AML-2-23). Thus, even if the Mo2 preparation had contained IgG antibodies binding to monocyte IgG Fc receptors, it is unlikely that the addition of the IgM specific secondary antibody would have induced a calcium response or H_2O_2 production in the cells. Third, a calcium flux was seen by using purified F(ab')$_2$ fragments of the CD14 mAb MEM15 and GAM-HL. This antibody also induced a calcium flux in a subset of granulocytes indicating related function of this molecule in different cell types. The failure of Mo2 to induce a calcium flux in granulocytes may be due to low expression of the Mo2 binding epitope on resting granulocytes [12]. The negative results obtained with mAbs binding to other myeloid antigens further support the view of an intrinsic signal transducing capacity of CD14.

It remains to be seen whether the demonstrated calcium mobilizing capacity of CD14 reflects a role for this antigen as a leukocyte receptor. In several regards CD14 resembles the FcRIII (CD16) on granulocytes. Both molecules are present in high density on mature cells [14]. They are linked to the cell membrane by phosphatidylinositol [3,14], their expression is subject to considerable regulation during cell activation [15,16] and both are found in soluble form in body fluids [4,17]. Thus, anatomical and physiological similarities with a receptor molecule, as well as gene mapping to a chromosome area encoding for receptors and growth factors [18], is in concordance with in vivo signal mediation through CD14. The nature of an eventual ligand and the biological significance of the signal transduction through CD14 can only be speculated. As the molecule is preferentially expressed on mature myeloid cells, a role in cell function rather than in the regulation of myeloid differentiation seems likely. The induction of H_2O_2 production as demonstrated by increased rhodamine-123 fluorescence following crosslinking of Mo2 and the stimulatory effect of CD14 mAbs on monocyte chemiluminescence during phagocytosis of zymosan [6] also support this view.

Two other PI-linked molecules, CD16 and CD24, have recently been shown to play a role in human leukocyte signal mediation [7,8]. Signal transduction through a third PI-linked leukocyte molecule, CD14, may suggest that the PI-anchor is facilitating signal transduction by cell surface molecules. As PI-linked molecules have high lateral mobility in the cell membrane [19], they may be more easily perturbed or aggregated upon interaction with a specific ligand. In addition, PI-linked molecules can be shedded from the cell membrane by enzyme hydrolysis [19], allowing control of cell activation by negative feedback. These may be properties making CD14 and other PI-linked antigens suited for signal mediation.

Acknowledgements: This study was supported by Wilfred Pettersens Legat, and grants from Inger-Margrethe and Per Jæger.

REFERENCES

[1] Todd III, R.F., Nadler, L. and Schlossmann, S.F. (1981) J. Immunol. 126, 1435–1442.
[2] Goyert, S.M., Tesio, L., Ashmann, L.K., Ball, E., Bazil, V., Garrido, F., Hogg, N., Horejsi, V., Howell, A., Jarayam, Y., Look, A.T., Ruiz-Cabello, F., Schmitt, D., Schneider, M. and Van der Schoot, C.E. (1989) in: Leukocyte Typing IV (Knapp, W. ed) pp. 789–794, Oxford University Press, New York.
[3] Simmons, D.L., Shencao, T., Tenen, D.G., Nicholson-Weller, A. and Seed, B. (1989) Blood 73, 284–289.
[4] Bazil, V., Horejsi, V., Baudys, M., Kristofova, H., Strominger, J.L., Kostka, W. and Hilgert, I. (1986) Eur. J. Immunol. 16, 1583–1589.
[5] MacIntyre, E.A., Roberts, P.J., Jones, H.M., Van der Schoot, E.C., Favalaro, J., Tidman, N. and Linch, D. (1989) J. Immunol. 142, 2377–2383.
[6] Schneider, E.M., Lorenz, I., Kögler, G. and Wernet, P. (1989) in: Leukocyte Typing IV (Knapp, W. ed) pp. 794–796, Oxford University Press, New York.
[7] Perussia, B., Cassatella, M.A., Anegon, I. and Trinchieri, G. (1989) in: Leukocyte Typing IV (Knapp, W. ed.) pp. 590–595, Oxford University Press, New York.
[8] Fischer, G., Majdic, O., Gadd, S. and Knapp, W. (1990) J. Immunol. 144, 638–641.
[9] Knapp, W. (ed) (1989) Leukocyte Typing IV, Oxford University Press, New York.
[10] Kao, J.P.Y., Harootunian, A.T. and Tsien, R.Y. (1989) J. Biol. Chem. 264, 8179–8184.
[11] Rothe, G., Oser, A. and Valet, G. (1988) Naturwissenschaften 75, 354–355.
[12] Jarayam, Y. and Hogg, N. (1989) in: Leukocyte Typing IV (Knapp, W. ed) pp. 796–797, Oxford University Press, New York.
[13] Loken, M.R., Shah, V.O. and Civin, C.I. (1987) in: Leukocyte Typing III (McMichael, A.J. ed) pp. 630–636, Oxford University Press, Oxford.
[14] Selvaraj, P., Rosse, W.F., Silber, R. and Springer, T.A. (1988) Nature 333, 565–567.
[15] Firestein, G.S. and Zvaifler, N.J. (1987) Cell. Immunol. 104, 343–354.
[16] Huizinga, T.W.J., Van der Schoot, C.E., Jost, C., Klaassen, R., Kleijer, M. and Von dem Borne, A.E.G.K. (1988) Nature 333, 667–669.
[17] Khayat, D., Geffrier, C., Yoon, S., Scigliano, E., Soubrane, C., Weil, M., Unkeless, J.K. and Jaquillat, C. (1987) J. Immunol. Methods 100, 235–241.
[18] Goyert, S., Ferrero, E., Rettig, W.J., Yenamandra, K., Obata, F. and Le Bau, M.M. (1988) Science 239, 497–500.
[19] Low, M.G. and Saltiel, A.R. (1988) Science 239, 268–275.