Brazilian Journal of Medical and Biological Research (1998) 31: 793-797
ISSN 0100-879X Short Communication

Precipitated immune complexes of IgM induce the generation of reactive oxygen species by rabbit polymorphonuclear leucocytes

Y.M. Lucisano[1], A.M. de-Mello[2], E.R. Vasques[2] and B. Mantovani[2]

[1]Departamento de Física e Química, Faculdade de Ciências Farmacêuticas, and [2]Departamento de Bioquímica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Abstract

We report that immune complexes of IgM (ICIgM) antibodies and ovalbumin in the form of a precipitate from the equivalence zone induce the generation of reactive oxygen species by rabbit blood polymorphonuclear leucocytes (PMN), as measured by the chemiluminescence (CL) production in the presence of luminol. The kinetics of CL generation induced by ICIgM is quite different from that induced by precipitated immune complexes of IgG (ICIgG): the maximum rate of CL production for ICIgM occurs around 14 min, whereas for ICIgG it occurs about 5 min after incubation with the cells. Also the triggering of the process requires a higher concentration of ICIgM than of ICIgG. Evidence is presented that these effects are not mediated by interaction of the antigen (ovalbumin) with the cell, since immune precipitates of ovalbumin and the $F(ab')_2$ fragment had no effect. Our observations that precipitated ICIgM can also be an effective stimulus for CL generation and thus for O_2^- production reveal a new functional capability of PMN. These results may have implications for the understanding of the participation of ICIgM (as well as of ICIgG) in inflammatory reactions mediated by PMN in immune complex diseases, and in the mechanisms of defense against microbes and other non-self agents.

Correspondence
Y.M. Lucisano
Departamento de Física e Química
FCF, USP
14049-900 Ribeirão Preto, SP
Brasil
Fax: 55 (016) 633-1092

Research supported by CNPq and FAPESP.

Received July 25, 1997
Accepted March 18, 1998

Key words
- Reactive oxygen species
- Polymorphonuclear leucocytes
- Immune complexes
- IgM
- IgG

The interaction of immune complexes (antigen-antibody complexes) with phagocytic cells may trigger a set of effector functions such as phagocytosis of the immune complexes, stimulation of lysosomal enzyme release and the respiratory burst with the production of highly reactive oxygen species such as O_2^- (superoxide anion), H_2O_2, $OH^{\bullet}$ (hydroxyl radical), and singlet oxygen (1,2). Normally such effector functions constitute an important component of the mechanisms of defense, promoting the killing of bacteria, fungi, parasites and tumor cells; but in some circumstances they may be involved in inflammatory reactions leading to tissue injury (3).

It might be expected that the cellular production of reactive oxygen species, with their potentially beneficial and deleterious effects, is very carefully regulated by the

cell, including a precise mechanism of recognition as to what kind of substances is inducing the cell activation. Certainly a primary event in this mechanism must be the binding of the stimulant molecule to the cell membrane with the subsequent triggering of the biochemical reactions for superoxide production.

In polymorphonuclear leucocytes (PMN), immunological receptors for IgG (Fcγ) and for IgA (Fcα) have been implicated in the initiation of the respiratory burst, since immune complexes of these classes can trigger the process. Also, complement receptors (for C3b, C3bi and C5a) may play a role in this phenomenon, albeit activation by C3b and C3bi is controversial (4). As to IgM immune complexes (ICIgM), there are so far no reports on their possible role as stimulating agents of the generation of reactive oxygen species in polymorphonuclear leucocytes.

Our group has shown that precipitated immune complexes of IgM antibodies and ovalbumin prepared from the equivalence zone were able to bind to rabbit PMN and stimulate the liberation of lysosomal enzymes, as well as immune complexes of IgG (ICIgG) (5-7). These findings, together with the fact that ICIgM have been found deposited in some tissues in immune complex diseases, have led us to investigate the possibility of ICIgM inducing the production of reactive oxygen species in rabbit blood polymorphonuclear leucocytes in a comparative study with the effect of ICIgG, both in the form of a precipitate from the equivalence zone.

The preparations of the antigen (ovalbumin), of rabbit IgG and IgM anti-ovalbumin antibodies as well as the isolation of rabbit blood PMN are described in reference 6. The chemical purity of IgG and IgM was tested by immunoelectrophoresis; in addition, any eventual contamination of the IgM preparation with IgA was excluded by immune diffusion tests with a goat antibody to rabbit IgA, α-chain-specific, obtained from Sigma Chemical Co. (St. Louis, MO).

To prepare $F(ab')_2$ antibody fragments, rabbit IgG anti-ovalbumin was hydrolyzed with pepsin (Sigma) as described in reference 8. The absence of uncleaved IgG in the $F(ab')_2$ preparation was confirmed by SDS-PAGE. Soluble collagen was purified from guinea pig skin as reported in reference 9.

The immune complexes were prepared as described in reference 6. Briefly, precipitin curves were obtained with purified IgG, IgM and $F(ab')_2$ antibodies and ovalbumin. The volumes of ovalbumin and antibody solutions were calculated from the point of equivalence in order to obtain the desired quantity of protein in the precipitates. Protein determinations were done by the microbiuret method (10).

PMN chemiluminescence (CL) was measured in a scintillation counter (LS-150 liquid scintillation system, Beckman Instruments Inc., Fullerton, CA) set in the out-of-coincidence mode with a fully open window (11). The reaction mixture consisted of 2×10^6 cells in a final volume of 2 ml in Hanks' balanced salt solution (HBSS) containing 0.1% gelatin, 100 mM luminol and the stimulus (precipitated immune complexes) in a glass scintillation counter vial. The vials had been kept overnight in the dark to avoid the excitation of glass fluorescence, and the preparation steps were performed in a dark room with a small red lamp. The mixture vials were incubated in a 37°C water bath and transferred to the counter (which was at room temperature) at predetermined time intervals for the measurement of the CL rate (counts per min; cpm) for 30 s, and immediately returned to the incubation bath.

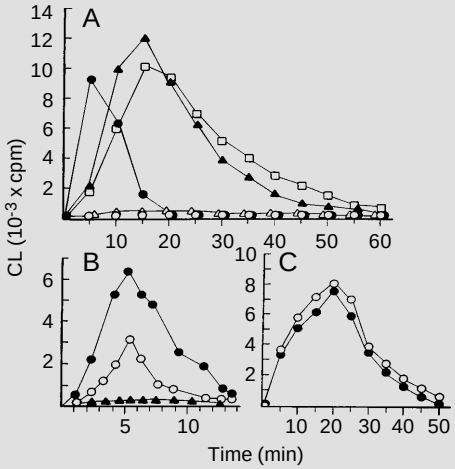
The experiments documented in Figure 1 show that ICIgM is able to induce the production of reactive oxygen species by rabbit PMN, as measured by the luminol-dependent chemiluminescence assay. The ability of PMN to trigger this function differs depending on the class of immune complex: with precipitated immune complexes at a concen-

tration of 100 µg protein/ml no effect is obtained with ICIgM, whereas ICIgG is quite effective even with half of this concentration (Figure 1A and B); however, with 200 µg protein/ml, ICIgM is very effective. Also, the time-courses of CL generation by these two classes of immune complexes are quite different: for ICIgG the CL response is relatively rapid, with a maximum rate around 5 min, while for ICIgM the effect develops more slowly and lasts longer, attaining the maximum rate around 14 min. IgM or IgG not complexed with the antigen at the same protein concentration as the immune complexes does not present any stimulation effect (data not shown). Figure 1C illustrates a control experiment for the effect of ICIgM which shows that there is practically no difference in CL production with this class of immune complex when the IgM antibody is prepared from pre-heated serum (56ºC for 30 min) to inactivate C1q or from normal serum. This was done to exclude a possible, although unlikely effect of C1q contamination of the IgM antibody preparation. Another experiment based on this possibility was also performed by testing the effect of soluble collagen (200 µg/ml) on the chemiluminescence induced by both classes of immune complexes and no inhibition was observed (data not shown). According to reported experiments (12), interaction mediated by C1q should be blocked by collagen.

In order to determine if the antigen (ovalbumin) present in the complexes could be responsible for the stimulation of the cells, we performed a control experiment using 200 and 400 µg/ml of the precipitated ICF(ab')$_2$ (which contains the antigen but lacks the Fc fragment). No chemiluminescence was generated and the profiles of the responses were similar to the control (data not shown).

Table 1 summarizes a series of experiments giving some kinetic parameters of CL production by PMN using both classes of immune complexes as stimulating agents at a concentration of 200 µg protein/ml; at this concentration the maximum rate of CL production induced by ICIgG is about double that produced by ICIgM.

The chemiluminescence produced by stimulated PMN in the presence of luminol



A - ○ PMN without any stimulus
· ICIgG, 100 µg/ml
△ ICIgM, 100 µg/ml
□ ICIgM, 200 µg/ml
▲ ICIgM, 300 µg/ml

B - · ICIgG, 100 µg/ml
○ ICIgG, 50 µg/ml
▲ PMN without any stimulus

C - · ICIgM, 200 µg/ml, IgM antibodies prepared from pre-heated serum (56ºC, 30 min)
○ ICIgM, IgM antibodies from non-heated serum

Figure 1 - Kinetics of chemiluminescence production by PMN stimulated with various concentrations of ICIgG and of ICIgM. The rate of CL generation in counts per min (cpm) of 2 x 10^6 PMN cells in 2 ml HBSS (with 0.1% gelatin and 10^{-7} M luminol) is measured in the course of time after incubation with precipitated immune complexes (concentrations expressed as µg total protein/ml). A, B and C represent independent experiments (different cell preparations).

Table 1 - Chemiluminescence produced by polymorphonuclear leucocytes stimulated with ICIgM and ICIgG.

[a]Time interval from the incubation of PMN and immune complexes (zero time) to the time of the maximum rate of CL emission. [b]Time interval from zero time to the time when the CL rate had decayed to 50% of the maximum. [c]Comparison of the maximum rates of CL, produced by ICIgG and ICIgM, taking 100 as the value for the CL induced by ICIgG and expressing the CL produced by ICIgM as the percentage in each paired experiment. Results are reported as means ± SD for the number within parentheses of independent experiments (different cell populations). Concentration of both classes of immune complexes: 200 µg/ml.

Stimulus	t_{max} (min)[a]	t_{50} (min)[b]	% rate of CL in t_{max}[c]
ICIgG	5.3 ± 0.3 (4)	9.8 ± 2.8 (3)	100
ICIgM	13.9 ± 3.5 (7)	26.3 ± 7.3 (7)	48 ± 3.4 (3)

depends on the formation of reactive oxygen species by the cell. The light emission occurs as a result of a series of steps, which can be described in general terms as follows (13): formation of superoxide (O_2^-) followed by its dismutation into H_2O_2 which, together with Cl^- and upon the action of a myeloperoxidase, produces OCl^-. Hypochlorite is capable of oxidizing luminol with the generation of an excited aminophthalate anion which relaxes to the ground state with the emission of light. Thus, the measurement of CL reflects the global effect depending on the rate of production of reactive oxygen species and on myeloperoxidase activity.

Many kinds of stimuli can induce the respiratory burst in phagocytes, leading to activation of NADPH oxidase, increased O_2 consumption and formation of superoxide; according to the type of stimulus, different signal transduction pathways may be involved (14,15). With regard to immune complexes, most studies have been done with the IgG class (4); from the available data (11,16) it appears that the kinetics of chemiluminescence is variable depending on the form in which the immune complexes (or the aggregated immunoglobulins) are presented to the cells (soluble, precipitated or adsorbed on surfaces). With precipitated ICIgG from the equivalence zone we observed a chemiluminescence effect which was limited in time, with a sharp peak around 5 min, in agreement with results reported for human neutrophils (16). Our observations that precipitated ICIgM can also be an effective stimulus for chemiluminescence generation and thus for O_2^- production reveal a new functional capability of PMN. The time-course of CL produced by this class of immune complex is quite different from that induced by ICIgG, indicating that the triggering and/or the biochemical pathways might be different. In previous experiments we had shown that ICIgG is readily phagocytosed by PMN whereas ICIgM remains mostly on the cell surface (5); thus, precipitated ICIgM seems to be an immunological stimulus for the respiratory burst not associated with phagocytosis. This may be related to the observed kinetic differences with ICIgG; another factor might be a slower rate of binding of ICIgM to the cells.

The presence of a cell membrane receptor for IgM in PMN is a controversial issue. Positive as well as negative evidence has been reported, as discussed previously (5). Possibly, these conflicting results may be related to the affinity of the binding sites on the cell membrane (receptor) for IgM and the different forms of presentation of the immunoglobulin to the cell. The fact that ICIgM in the form of a precipitate can bind to PMN and induce lysosomal enzyme release (5-7) as well as trigger superoxide anion production, as we have shown, may be dependent on the cooperative action of a large number of weak interactions with a low affinity membrane receptor, favored by the size of this kind of immune complex. This agrees with some studies with human neutrophils, using myeloma immunoglobulins, showing that unaggregated IgM binds very poorly to cells, but the binding was considerably increased when the IgM molecules had been aggregated with the $F(ab')_2$ fragment of anti-human immunoglobulin (17). It also confirms some observations showing the effective binding of IgM immune complexes to human neutrophils using chimeric mouse-human IgM antibodies (18). Thus, the recognition of immune complexes by PMN mediated by IgM would require the clustering of many immunoglobulin molecules, the interaction being non-competitive with fluid phase IgM, as observed previously (5). The triggering of superoxide production and lysosomal enzyme release may be related to the cross-linking of the receptors upon interaction with the immune complex, as reported for IgG Fc receptors in human neutrophils (19). The possibility that changes in IgM conformation when forming the immune complex might expose or form

the binding site should also be considered. Although the subject is controversial, some evidence points toward conformational changes occurring upon the combination of antibodies with antigen, but at present this evidence is restricted to experiments performed with the Fab fragment of IgG (20).

Whatever the molecular mechanism of interaction of ICIgM with PMN, the finding that this class of immune complexes can trigger effector functions of these cells (lysosomal enzyme release and production of reactive oxygen intermediates) must have physiological as well as physiopathological implications. ICIgM as well as ICIgG have been found deposited in various tissues in immune complex diseases (21,22), and thus they can be one of the recognition signals for neutrophils to produce tissue damage, a divergent function from the normal mechanism of defense against microbes and other non-self agents.

Acknowledgments

The technical assistance of Silvana Chedraoui Silva and José Antonio da Silva and the secretarial help of Maria Thereza Rodrigues and Maria Regina de P. Raphaloski are gratefully acknowledged.

References

1. Segal AW (1989). The respiratory burst in monocytes and macrophages. In: Zembala M & Asherson GL (Editors), *Human Monocytes.* Academic Press, London, 89.
2. Malech HL & Gallin IJ (1987). Neutrophils in human diseases. *New England Journal of Medicine*, 317: 687-694.
3. Fantone JC & Ward PA (1986). Polymorphonuclear leukocyte-mediated cell and tissue injury: oxygen metabolites and their relations to human disease. *Human Pathology*, 16: 973-978.
4. Seifert R & Schultz G (1991). The superoxide-forming NADPH oxidase of phagocytes. *Reviews of Physiology, Biochemistry and Pharmacology*, 117: 70-126.
5. Furriel RPM, Lucisano YM & Mantovani B (1992). Precipitated immune complexes of IgM as well as of IgG can bind to rabbit polymorphonuclear leucocytes but only the immune complexes of IgG are readily phagocytosed. *Immunology*, 75: 528-534.
6. Lucisano YM & Mantovani B (1984). Lysosomal enzyme release from polymorphonuclear leukocytes induced by immune complexes of IgM and of IgG. *Journal of Immunology*, 132: 2015-2020.
7. Lucisano YM & Mantovani B (1988). The role of complement in the stimulation of lysosomal enzyme release by polymorphonuclear leucocytes induced by immune complexes of IgG and of IgM. *Immunology*, 65: 171-175.
8. Nisonoff A (1964). Enzymatic digestion of rabbit gamma globulin and antibody and chromatography of digestion products. In: Eisen HN (Editor), *Methods in Medical Research*. Vol. 10. Year Book Medical Publishers, Inc., Chicago, 134.
9. Epstein Jr EH, Scott RD, Miller EJ & Piez KA (1971). Isolation and characterization of the peptides derived from soluble human and baboon skin collagen after cyanogen bromide cleavage. *Journal of Biological Chemistry*, 246: 1718-1724.
10. Itzhaki RF & Gill DM (1964). A micro-biuret method for estimating proteins. *Analytical Biochemistry*, 9: 401-410.
11. Cheung K, Archibald AC & Robinson MF (1984). Luminol-dependent chemiluminescence produced by neutrophils stimulated by immune complexes. *Australian Journal of Experimental Biology and Medical Science*, 62: 403-419.
12. Tenner AJ & Cooper NR (1980). Analysis of receptor-mediated C1q binding to human peripheral blood mononuclear cells. *Journal of Immunology,* 125: 1658-1664.
13. DeChatelet LR, Long GD, Shirley PS, Bass DA, Thomas MJ, Henderson FW & Cohen MS (1982). Mechanism of the luminol-dependent chemiluminescence of human neutrophils. *Journal of Immunology*, 129: 1589-1593.
14. Crockett-Torabi E & Fantone JC (1990). Soluble and insoluble immune complexes activate human neutrophil NADPH oxidase by distinct Fcγ receptor-specific mechanisms. *Journal of Immunology*, 145: 3026-3032.
15. Blackburn Jr WD & Heck LW (1989). Neutrophil activation by surface bound IgG is via a pertussis toxin insensitive G protein. *Biochemistry and Biophysics Research Communications*, 164: 983-989.
16. Klauser RJ, Schmer G, Chandler WL & Müller W (1990). Consumption of complement and activation of human neutrophils by an artificial immune complex: polyacrylic acid-IgG polymer. *Biochimica et Biophysica Acta*, 1052: 408-415.
17. Lawrence DA, Weigle WO & Spiegelberg HL (1975). Immunoglobulins cytophilic for human lymphocytes, monocytes and neutrophils. *Journal of Clinical Investigation*, 55: 368-387.
18. Zhang W & Lachmann PI (1996). Influence of antibody class and complement on neutrophil endocytosis of defined immune complexes. *Molecular Immunology*, 33 (Suppl 1): 60-68.
19. Willis HE, Browder B, Feister AJ, Mohanakumar T & Ruddy S (1988). Monoclonal antibody to human IgG Fc receptors. Cross-linking of receptors induces lysosomal enzyme release and superoxide generation by neutrophils. *Journal of Immunology*, 140: 234-239.
20. Rini JM, Schulze-Gahmen U & Wilson IA (1992). Structural evidence for induced fit as a mechanism for antibody-antigen recognition. *Science*, 255: 959-965.
21. Cohen AH, Border WA, Fong H, Glassock RJ & Trygstad C (1982). Glomerulonephritis with mesangial IgM deposits. *Kidney International*, 21: 147-154.
22. Tejani A & Nicastri AD (1983). Mesangial IgM nephropathy. *Nephron*, 35: 1-5.