Reduced receptor-mediated processing of model immune complexes by macrophages from patients with rheumatoid arthritis and its regulation by cytokines

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The present study was conducted to understand the role of peripheral blood-derived monocytes in the pathogenesis of rheumatoid arthritis (RA), their contribution in relation to the receptor-mediated processing of circulating immune-complexes (ICs) and the phenotypic and functional changes they undergo due to the abundant cytokines. In this study we have checked the expression of FcRIII (CD16), CR1 and CR3 on the cultured monocytes of RA patients and their phagocytic activity by flow cytometry. Significantly reduced expression of FcRIII, CR1 and CR3 was observed on the monocyte-derived macrophages of RA patients. Phagocytosis of soluble ICs both in the absence and presence of complement was reduced in RA patients. Significant direct relationship was observed between FcRIII expression and FcR-mediated phagocytosis as well as between CR1 expression and complement-mediated phagocytosis, indicating impaired receptor-mediated processing in RA patients. Effect of three cytokines, TNF-β, IL1-α and IFN-γ on the receptor expression and phagocytic activity was also determined. Our results suggest that receptor expression and phagocytic activity of monocyte population in RA is affected by the existing cytokine milieu.

RHEUMATOID arthritis (RA) is a common human autoimmune disease which is characterized by a chronic inflammation of the synovial joints and infiltration by blood-derived cells, chiefly memory T cells, macrophages and plasma cells. The role of T and B cells in the pathogenesis of RA is well established. However, less attention has been focused on monocytes and cells of the fixed macrophage system in RA and their role in the disease pathogenesis.

Ingestion of pathogens by phagocytic cells is a central process in host defence against infection. Mononuclear phagocytes express several surface receptors that mediate phagocytosis of invading pathogens including Fc receptors and the mannose–fucose receptor. FcRIII (CD16) has been proposed as the Fc receptor that participates in immune-complex (IC) binding. However, of particular importance are the complement receptors 1 (CR1) and 3 (CR3) which bind the C3 fragments C3b and iC3b, respectively. The major processing of ICs in the fixed macrophage system occurs with the cooperative action of Fc receptor and complement receptors. The role of cytokines and ICs has been suggested by Quayle et al. in the induction of receptor expression on neutrophils. Whether the receptor expression on monocytes and macrophage populations in RA is also affected by the existing cytokine milieu is yet to be confirmed. While cytokines such as IFN-γ, TNF and GM-CSF are known to activate other macrophage functions such as expression of MHC antigens and microbicidal or tumoricidal activity, their effect on complement receptor expression and complement dependent phagocytosis has not been extensively examined.

Our preliminary studies have shown that complement proteins, complement breakdown products and complement regulatory proteins are correlated with disease activity in patients with RA. This study investigates the expression of FcRIII and complement receptors on monocyte-derived macrophages, phagocytic activity of these macrophages and effect of cytokines on the receptor expression and phagocytosis. The processing of both plain and C3b opsonized heat aggregated ICs was studied to examine the status of Fc receptor (FcR) and complement receptor (CR) mediated binding. Finally, receptor expression was correlated with phagocytosis in order to establish that impaired clearance is due to the reduced receptor expression.

Twenty-five patients (seven males and eighteen females, mean age 44.6 years, range 25–62 years) were selected from the Rheumatology Clinic of the All India Institute of Medical Sciences (AIIMS). All the patients selected fulfilled the American Rheumatism Association (ARA) revised criteria for classification of RA. Criteria for entry into the study included RA of more than 6 months duration, with onset after 16 years of age and at least 3 of the following, ≥ 3 swollen joints, ≥ 6 tender joints, ≥ 20 min of morning stiffness and Westergren erythrocyte sedimentation rate (ESR) of ≥ 30 mm/h. There was no renal or skin involvement in any of the above-mentioned patients. Rheumatoid factor (RF) was estimated by latex fixation test (LFT) using Rapi Tex-RF supplied by Behringwerke AG, Germany. LFT was considered positive when agglutination was present in more than 1 : 12 dilution of serum corresponding to 40 U/ml. Only RF positive patients were included in the study. Blood samples were collected before starting the treatment. The normal population (fifteen males and ten females, mean age 31.5 years, range 19–45 years) consisted of laboratory personnel and non-laboratory volunteers, all having no history of rheumatic diseases.

Fluorescein isothiocyanate (FITC), human globulin (Cohn's fraction II and III), histopaque (density 1.077 g/ml), RPMI-1640, Hank's balanced salt solution (HBSS), IL1-α and TNF-β were obtained from Sigma Chemical Co., USA. Foetal calf serum (FCS) was obtained from

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GIBCO-BRL, USA. Mouse monoclonal immunoglobulins to CR1, CR3 and CD16 and FITC conjugated rabbit anti-mouse IgG were obtained from Dakopatts Diagnostics, Denmark. IFN-γ was a generous gift from Naveen Khanna, ICGEB, India.

Mononuclear cells were obtained from heparinized blood by density gradient centrifugation on Ficoll-Hypaque, at a density of 1.080 g/cc. After centrifugation (600 g for 30 min at 20°C), the mononuclear cell layer was washed 3 times in fresh RPMI-1640 and then suspended in RPMI containing 10% heat inactivated sera. The cells were then plated in culture dishes and incubated for 1 h at 37°C. At the end of 1 h, non-adherent cells were removed and adherent cells were cultured with different stimulants.

Human IgG was conjugated with FITC as described by Johnson et al.9. Conjugation was carried out at a final protein concentration of 10 mg/ml at pH 9. This was accomplished by dilution of IgG to an appropriate volume using isotonic saline solution and carbonate–bicarbonate buffer, pH 9. IgG solution was chilled in an ice-bath before adding the dry FITC powder. 0.05 mg of FITC was added per 1 mg of protein. The mixture was then kept at 4°C for 18 h with gentle mixing to avoid frothing. The solution was then dialysed against several changes of PBS pH 7. Both the labelled and unlabelled monomeric IgG were dissolved in 0.01 M, pH 7.4 PBS and the mixture centrifuged at 1200 g for 10 min to remove any visible pellet. The supernatant was aggregated by heating at 63°C for 20 min. The larger aggregates were then removed by centrifugation at 1200 g and layered on a sucrose density gradient (10–30%) and centrifuged at 1,000,000 g for 3 h at 4°C. The different-sized aggregates were separated based on the difference in their densities using a fraction collector. The aggregates composed of about 20–30 molecules of IgG were used in the experiments. The oposized complexes bearing approximately 20 molecules of C3b/AIgG were prepared based on the method of Daha and Van Es10.

Monocyte-derived macrophages were incubated with fluorescent labelled AIgG with rotational agitation at 37°C for 30 min in a shaking water bath and then 2 ml of 3 mM ethylene diamine tetraacetic acid (EDTA) was added in order to terminate phagocytosis. After washing the cells were resuspended in PBS and were applied to flow cytometer (Coulter, Epics-XL). Quantification of phagocytic activity was estimated by mean fluorescence per cell. It was determined using the following formula:

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\text{Sum of} \left( \frac{\text{cell count on each channel} \times \text{channel number}}{\text{Total cell count}} \right)
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Macrophages were suspended in PBS–BSA buffer at 1 x 10⁶ cells and incubated for 1 h with mouse anti-CR1, anti-CR3 and anti-CD16 antibody. After washing with PBS–BSA buffer, cells were incubated with FITC labelled anti-mouse antibody. The cells were analysed using a flow cytometer (Coulter) after fixing in paraformaldehyde.

The amount of FITC-MoAb bound to CR1, CR3 and CD16 was determined from the means of fluorescence intensity.

Comparison between groups was performed by calculating confidence interval (CI) for the means using t-test. The correlations between the biological parameters were evaluated by linear regression analysis, using Student's t-test to determine whether the correlation was significant.

The phagocytic activity was investigated in blood-derived 1-, 3-, 5- and 7-day-old monocytes using both plain and opsonized labelled complexes. An enhancement in both FcR and CR-mediated phagocytosis was observed with the differentiation of monocytes to macrophages. Maximum fold enhancement in phagocytic activity was observed on the 5th day.

The phagocytosis of plain and opsonized complexes was checked in blood-derived 5-day-old macrophages. There was a significant reduction in the FcR and CR-mediated phagocytosis in RA patients by 47 and 44%, respectively, when compared to the phagocytosis by normal macrophages (P < 0.001) (Figure 1). An enhancement

![Figure 1. Phagocytic activity of monocyte-derived macrophages in the absence of complement, mediated by Fc receptors (●) as well as in the presence of complement, mediated by Fe + C receptors (+). 5-day-old macrophages from normal (N) controls and rheumatoid arthritis (RA) patients were incubated with fluorescent labelled plain and opsonized aggregates (25 fluorescein) at 37°C for 30 min. Mean fluorescence intensity was determined by acquiring the cells in flow cytometer. Mean phagocytic activity (fluorescence) of normal macrophages was 89.18 ± 6.14 (CR-mediated) and 71.71 ± 6.59 (FcR-mediated) whereas that of RA macrophages was 49.48 ± 7.53 (CR-mediated) and 38.5 ± 8.45 (FcR-mediated).](image-url)
of 25% was observed in CR-mediated phagocytosis compared to FcR-mediated phagocytosis when normal macrophages were incubated with opsonized complexes (P < 0.001) whereas 29% enhancement in CR-mediated phagocytosis was observed when RA macrophages were incubated with opsonized complexes (P < 0.001).

To check whether reduction in phagocytosis is due to the reduced expression of receptors on the macrophages, CD16, CR1 and CR3 expression was studied on the monocyte-derived macrophages of normal and RA patients on the 5th day. Cells were incubated under the conditions of saturation and equilibrium with anti-CD16, anti-CR1 and anti-CR3. The amount of bound monoclonal antibodies was quantitated with fluoresceinated anti-mouse immunoglobulins by flow cytometry. Monocyte population was gated using anti-CD14-45 antibody. Expression of all the three receptors was reduced on the macrophages of RA patients. In RA patients, mean fluorescence of CD16 was 51% of CD16 on macrophages from normal individuals (P < 0.001). The mean number of CR1 on RA macrophages was 48% of that on normal macrophages whereas mean number of CR3 on RA macrophages was 62% of that on normal macrophages (P < 0.001) (Figure 2).

To examine whether pro-inflammatory cytokines produced abnormally in RA patients play any role in the expression of cell surface receptors and phagocytic activity of macrophages, monocytes were cultured in the presence of cytokines. Mean fluorescence of CR1 and CR3 expression on the macrophages in the presence of media only was 20 ± 3.2 and 24.9 ± 3.3, respectively whereas CR-mediated phagocytic activity in the presence of media was 49.4 ± 2.3. TNF-β (100 U/ml) enhanced the complement-mediated phagocytosis (85%, P < 0.001) as well as CR1 (83%, P < 0.001) and CR3 (97%, P < 0.001) expression whereas IL1-α (100 U/ml) reduced the complement-mediated phagocytosis (45%, P < 0.001) as well as CR1 (25%, P < 0.01) and CR3 (59%, P < 0.001) expression (Figure 3). Mean fluorescence of expression of FcRIII on the macrophages in the presence of media was 19.9 ± 4.3 whereas FcR-mediated phagocytic activity in the presence of media was 38.5 ± 2.2. Both TNF-β and IL1-α had no effect on FcR-mediated phagocytosis and FcRIII expression on macrophages (Figure 4). Cell surface expression of CR1 was down regulated by 38% (P < 0.01) in response to IFN-γ (100 U/ml) whereas CR3 expression and CR-mediated phagocytosis were not affected by IFN-γ. IFN-γ enhanced the FcRIII expression and FcR-mediated phagocytosis by 53% (P < 0.001) and 40% (P < 0.001), respectively.

The relationship between the expression of receptors on macrophages and phagocytic activity of macrophages was investigated. A statistically significant positive relationship was observed between FcRIII expression and FcR-mediated phagocytosis (r = 0.87, P < 0.001, n = 25) as well as between CR1 expression and CR-mediated phagocytosis (r = 0.64, P < 0.001, n = 25).

Human peripheral blood monocytes appear to leave the circulation and migrate into various tissues where they become fixed tissue macrophages through a process of adaptive differentiation. The properties of these mature
macrophages depend on their local environment and these monocyte-derived macrophages play a critical role in host defence. Much attention has been addressed to both in vivo and in vitro studies of the function of monocytes and macrophages in systemic lupus erythematosus (SLE). Monocytes from SLE patients ingested or bound erythrocytes-AlgG to a higher degree than monocytes from normal controls. In this study, we have concentrated on the functional aspects of macrophages in RA. The FcR and CR-mediated phagocytosis of FITC labelled ICs by monocytes differentiated under in vitro culture conditions to macrophages was investigated.

We observed abnormally low phagocytosis in RA patients compared to normal subjects as already suggested by Williams and Lockwood. The results of this study are also in line with recent in vivo studies on mononuclear phagocyte function in patients with RA. Decreased clearance of soluble immune aggregates from the circulation was shown by Lobatto et al. In contrary to these reports, Hoch and Schur found enhanced antibody-coated sheep red blood cell rosette formation and phagocytosis by RA monocytes, while others reported normal phagocytosis by RA monocytes. Since the etiology of RA is elusive it is very difficult to explain these contradictory reports regarding phagocytic activity in RA patients from different population groups. To the best of our knowledge there is no other report on the phagocytic activity of macrophages in RA patients from Indian population.

The complement receptor-mediated processing is very significant for the elimination of ICs from the circulation especially in RA patients where high circulating ICs are a characteristic feature. An efficient clearance can be mediated by a cooperative processing by Fc and complement receptors (CR1 and CR3). We observed a reduced CR-mediated phagocytosis by the monocytes from RA patients which indicates that there is an impaired IC clearance in RA patients. It may be that ICs, which are not phagocytosed by the MPS, precipitate in other parts of the body (vessel wall) and cause inflammatory reactions. These findings suggest that certain properties of RA monocytes differ from the normal and may contribute to the pathogenesis of the disease.

It has been established from our study that monocytes from patients with RA have diminished capacity to phagocytose AlgG in the absence as well as in the presence of complement. To determine the role their membrane receptors play in the pathogenesis of RA, we checked the expression of FeRIII, CR1 and CR3 on the cultured monocytes using flow cytometry. We observed highly reduced expression of all the three receptors on the monocytes of RA patients compared to the normal monocytes. Decreased expression of CR1 and CR3 on monocytes of RA patients has also been shown by Heurkins et al. A study conducted by Jones et al. showed that CR1 and CR3 expression on peripheral blood polymorphonuclear cells (PMN) from patients with RA was significantly reduced compared to the normal controls. In contrary to these reports, Torsteinsdottir et al. showed that expression of CR1 and CR3 on the monocytes was elevated in RA patients. Although the underlying mechanism of the decreased FeRIII, CR1 and CR3 expression is not entirely clear, yet the most pertinent explanation for such a finding is a disease or inflammation-induced impairment of the receptor expression. Other possible reasons may be a rapid turnover of the receptors, which may overcome the normal turnover rate leading to a reduced presence of the receptor on the membrane. High cytokine levels like pro-inflammatory cytokines associated with RA can also down-regulate the receptor expression. Further, like in SLE, where the presence of autoantibodies to CR1 renders the receptor incapable of functioning as a receptor for C3b-coated complexes, such antibodies may block the receptor expression in this case too. A reduced complement receptor expression on erythrocytes of RA patients suggests that an overall reduction in CRs is influenced by the disease process.

Our study has identified functional defects of both Fc and complement-mediated phagocytosis in monocytes from patients with RA. In order to establish whether these abnormalities are due to defects in receptor expression we performed a simple linear regression analysis. Significant correlation between CR1 expression and phagocytosis of opsonized ICs by macrophages of patients with RA was observed which suggests that the diminished number of receptors expressed is at least partly responsible for the
described defect. Significant correlation was also observed between FcR-mediated phagocytosis and FcRRII expression, suggesting that FcRRII plays a major role in IC phagocytosis. In vivo experiments in chimpanzees have shown that monoclonal antibodies against FcRRII inhibit the elimination of E-IgG, suggesting that FcRRII may be the most important FcR that participates in IC binding. In another study, both FcRI and FcRRII were found to mediate binding and phagocytosis of erythrocytes.

RA patients have many pro-inflammatory cytokines and their blood concentration correlates with the severity of RA. This is compensated to some extent by the increased production of anti-inflammatory cytokines and cytokine inhibitors. To understand the role of cytokines in the pathogenesis of RA, we checked the effect of three cytokines, viz. IL-1α, TNF-β and IFN-γ on the receptor expression and phagocytic activity of monocytes. Expression of FcRRII and FcR-mediated phagocytosis was significantly increased in response to IFN-γ (refs 25, 26). Cell surface expression of CR1 was down-regulated whereas CR3 expression and CR-mediated phagocytosis were not affected by IFN-γ which suggests that IFN-γ may be involved in regulating the expression of cell surface receptors. TNF-β activated macrophages to phagocytose ICs by up-regulating the expression of complement receptors whereas IL1-α had an inhibitory effect. This suggests that phagocytic activity and cell surface expression on macrophages are regulated by various cytokines in a different manner in vitro. Within an inflammatory environment of RA, the net effect of cytokines depends on the balance between cytokines, inhibitors, cell surface receptors and their relative expression. Our findings suggest that selected activation and/or differentiation of blood-derived monocytes to cause the impaired phagocytosis may be generated not by a single cytokine but by several cytokines, which are involved in a macrophage-related cytokine network and analysis of cytokine regulation may yield effective therapeutic targets in inflammatory disease.


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