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Immunological detection of myeloperoxidase in synovial fluid from patients with rheumatoid arthritis

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We have used rocket immunoelectrophoresis and immunoblotting to detect myeloperoxidase in synovial fluid from patients with rheumatoid arthritis. This protein was enzymatically inactive but its identity as myeloperoxidase was confirmed by comparing its subunit structure with that of the purified enzyme. When neutrophils were stimulated to secrete myeloperoxidase *in vitro*, a polypeptide with an apparent molecular mass of 62 kDa was detected extracellularly by immunoblotting. Neutrophils isolated from synovial fluid showed a reduced level of this 62 kDa polypeptide but it was detected extracellulary in synovial fluid by immunoblotting. Thus, we conclude that neutrophils in synovial fluid from patients with rheumatoid arthritis have been activated *in vivo* to secrete myeloperoxidase and propose that the products of this enzyme system can contribute to the tissue damage associated with this disease.

INTRODUCTION

Polymorphonuclear leucocytes (neutrophils) are phagocytic cells of the immune system which function to protect the host from microbial infections. In order to perform this crucial role in host defence, they contain a battery of enzymes and associated pathways which can be utilized for cell killing and these may be broadly divided into O_2-independent (Elsbach & Weiss, 1983; Spitznagel, 1984) and O_2-dependent (Karnovsky & Bolis, 1982; Babior, 1978, 1984) mechanisms. In view of the facts that neutrophils are found in large numbers in synovial fluid of patients with rheumatoid arthritis and that they have the capacity to generate oxidants extracellularly, it has been proposed that the joint damage associated with this disease is attributable, at least in part, to oxidant generation by activated neutrophils (Halliwell *et al.*, 1985).

The generation of a full complement of reactive oxidants by neutrophils requires the activities of two enzyme systems. The first of these is an NADPH oxidase system which is responsible for the one-electron reduction of O_2 to generate superoxide radicals (Babior, 1978; Rossi, 1986); H_2O_2 formation may then occur by the spontaneous or enzymatic dismutation of superoxide radicals. The second enzyme is myeloperoxidase, a haemoprotein present in azurophilic granules of neutrophils (Klebanoff & Clark, 1978): during activation azurophilic granules release their contents at the site of oxidant generation so that myeloperoxidase can react with its substrate, H_2O_2, and thus generate HOCl (and related compounds) and possibly singlet O_2. Formation of hydroxyl radicals ($OH^{\cdot}$) may occur via reactions involving both superoxide radicals, H_2O_2 and a suitable transition metal salt catalyst, in a metal-catalysed Haber–Weiss-type reaction (Halliwell & Gutteridge, 1985, 1986). The high reactivities of both $OH^{\cdot}$ and HOCl are well documented.

It has recently been shown that iron salts in a form suitable to participate in $OH^{\cdot}$ production are detectable in some synovial fluid samples from patients with rheumatoid arthritis (Gutteridge *et al.*, 1982; Rowley *et al.*, 1984; Gutteridge, 1987). Therefore, considerable interest is focusing on the possible therapeutic role of iron chelators such as desferrioxamine in the treatment of this disease (Blake *et al.*, 1985; Polson *et al.*, 1985, 1986). However, the mere removal of transition metal salts to prevent $OH^{\cdot}$ formation will still leave the myeloperoxidase system operational and thus the possibility exists that the oxidizing products of this enzyme can evoke considerable tissue-damaging reactions. It was thus the aim of this work to establish whether neutrophils in synovial fluid from patients with rheumatoid arthritis had been activated *in vivo* to release myeloperoxidase in order to establish more clearly the molecular mechanisms responsible for joint degeneration in this disease.

EXPERIMENTAL

Isolation and purification of cells

Neutrophils were isolated from heparinized venous blood from healthy volunteers or patients with rheumatoid arthritis by a sedimentation procedure using M-PRM (Flow Laboratories) exactly as described previously (Edwards *et al.*, 1987*a*). Synovial fluid was collected by aspiration of knee joints and portions (1 ml) were centrifuged at 11600 *g* for 5 min and the cell-free supernatants obtained were stored at −20 °C until use (myeloperoxidase activity was stable when stored at −20 °C). The remaining synovial fluid was then diluted 2-fold with 0.9% NaCl, centrifuged at 1000 *g* for 5 min and the cell pellet was washed three times with 0.9% NaCl. Contaminating erythrocytes were lysed by hypo-osmotic lysis (Edwards & Swan, 1986) and after restoration of tonicity, cells were centrifuged over a cushion of Ficoll–Paque (Pharmacia) at 1000 *g* for 20 min. The purified neutrophils were washed twice and then suspended in a buffer containing (mM): NaCl, 120; KCl, 4.8; KH_2PO_4, 1.2; $CaCl_2$, 1.3; $MgSO_4$, 1.2; Hepes (pH 7.4), 25; 0.1% bovine serum albumin.

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Other methods

Proteins were separated by polyacrylamide-gel electrophoresis in 12.5% gels containing SDS (Laemmli, 1970) and then either stained with Coomassie Blue or transferred electrophoretically to nitrocellulose (Burnette, 1981). After transfer, nitrocellulose filters were incubated in phosphate-buffered saline containing 0.2% Tween 20 and primary antibody for 1 h at room temperature. After washing with several changes of phosphate-buffered saline/Tween, specifically-bound primary antibody was detected by incubation with horseradish peroxidase-linked swine anti-(rabbit IgG) antibody (Dako) for 1 h at room temperature: after several washes the filters were stained for peroxidase using 3,3′-diaminobenzidine. Rabbit anti-(human myeloperoxidase) antibody was raised by subcutaneous injections of human myeloperoxidase (Edwards & Swan, 1986), prepared by the method of Pember *et al.* (1983). Rocket immunoelectrophoresis was performed essentially as described previously (Laurell & McKay, 1981). Myeloperoxidase was assayed enzymatically by the guaiacol method (Bergmeyer, 1974). Suitable controls indicated that oxidatively-inactivated myeloperoxidase did not have altered immuno-reactivity.

RESULTS

Myeloperoxidase in synovial fluid

Of eight samples of synovial fluid from patients with rheumatoid arthritis originally screened, only one contained detectable levels of peroxidase activity, as determined by the ability of the fluid to oxidize guaiacol to tetraguaiacol (Table 1). However, it must be stressed that many peroxidases can oxidize guaiacol and thus this single positive result cannot be unequivocally assigned to myeloperoxidase activity. We have previously shown that when myeloperoxidase is released from activated neutrophils under conditions whereby oxidants are also released, the enzyme is rapidly oxidatively inactivated (Edwards *et al.*, 1987*b*) due to compound II formation (Edwards & Lloyd, 1986). Therefore, assays designed to measure myeloperoxidase activity under these conditions will (inevitably) grossly underestimate the amount of protein present. This problem may be overcome by utilizing immunologically-based assays using anti-(human myeloperoxidase) antibody. Thus, when the same synovial fluid samples were analysed for immunologically-detectable myeloperoxidase protein (rather than activity) by rocket immunoelectrophoresis, the protein was found to be present in all samples analysed, but at varying levels. Samples (analysed in triplicate) contained myeloperoxidase at levels ranging from 16–29 $\mu g \cdot ml^{-1}$. These results have since been confirmed in a further series of samples.

Protein profiles and immunoblot analysis of synovial fluid

In view of the fact that the synovial fluid contained immuno-reactive, but enzymatically inactive, myeloperoxidase, it was necessary to confirm the identity of this protein by analysing its molecular structure and comparing it with that of the purified enzyme.

The polypeptide profiles of the same eight synovial fluid samples analysed in Table 1 were then examined by polyacrylamide-gel electrophoresis in order to identify

Table 1. Myeloperoxidase in synovial fluid

Portions of synovial fluid from patients with rheumatoid arthritis (after removal of cells) were assayed for peroxidase by its ability to oxidize guaiacol to tetraguaiacol. The samples were then assayed for myeloperoxidase protein by rocket immunoelectrophoresis and quantified by comparing rocket areas with those obtained using dilutions of purified enzyme. N.D., non detected.

Sample number	Enzyme activity (munits/ml) (guaiacol oxidation)	Amount of protein (μg/ml) (rocket immunoelectrophoresis)
1	N.D.	16
2	N.D.	19
3	N.D.	17
4	N.D.	29
5	N.D.	18
6	4.2	23
7	N.D.	20
8	N.D.	20

polypeptide(s) which could be attributable to purified myeloperoxidase. The major polypeptides of synovial fluid had apparent molecular masses of 61–63, 57 and 51–53 kDa and only minor differences in polypeptide profiles and intensity of staining were observed in the different samples (Fig. 1*a*). The major subunit of purified myeloperoxidase in this range was 55 kDa (with less-well-defined bands at 39 kDa and 15 kDa).

Identical samples were then immunoblotted and myeloperoxidase visualized using primary antibody raised to the purified enzyme. Purified myeloperoxidase comprised two immuno-reactive subunits of apparent molecular masses of 55 and 63 kDa and all eight synovial fluid samples contained a polypeptide of 63 kDa which cross-reacted with the antiserum (Fig. 1*b*).

Polypeptide profiles and immunoblots of neutrophils stimulated *in vitro*

Neutrophils may be activated *in vitro* by the chemotactic peptide fMet-Leu-Phe plus cytochalasin B to secrete myeloperoxidase (Edwards *et al.*, 1987*b*). Therefore, the polypeptide profiles of non-activated and activated cells, together with released proteins, were compared and myeloperoxidase identified by immunoblotting. The polypeptide profiles of non-activated and activated cells were remarkably similar but in cells activated to secrete myeloperoxidase, polypeptides with apparent molecular masses of 74, 63, 55 and 39 kDa stained less intensely (Fig. 2*a*). Correspondingly, polypeptides with apparent molecular masses of 74, 63, 55 and 39 kDa (plus a few other bands) were found extracellularly after activation (Fig. 2*a*).

Identical samples were then immunoblotted and stained for immuno-detectable myeloperoxidase. Purified myeloperoxidase comprised immuno-reactive polypeptides with apparent molecular masses of 55 and 63 kDa while in both activated and non-activated neutrophils the same three polypeptides could also be distinguished, the 63 kDa band staining most intensely (Fig. 2*b*). In the cell-free supernatant from activated cells the 63 kDa polypeptide was clearly identified (Fig. 2*b*).

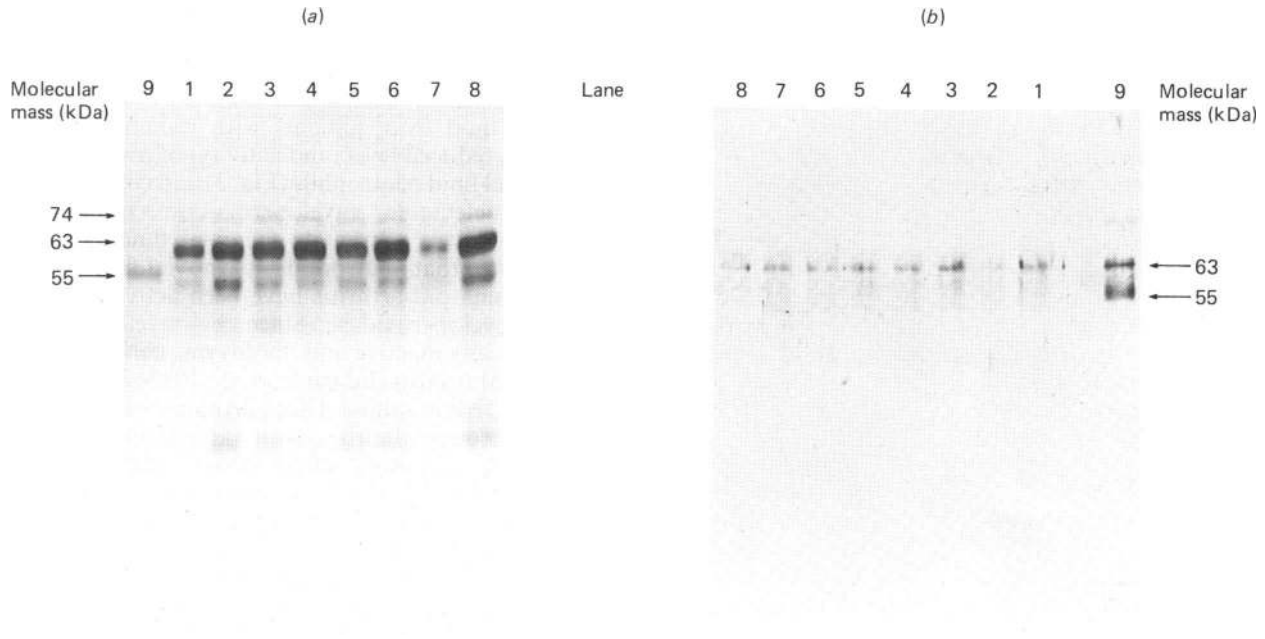

Fig. 1. Protein profiles and immunoblots of synovial fluid

Samples of synovial fluid (1 μl) were solubilized in SDS-sample buffer containing β-mercaptoethanol (99 μl) and heated to 90 °C for 2 min. Two 12.5% polyacrylamide gels were prepared and to one of these 25 μl of the above mix was added (lanes 1–8). After electrophoresis this gel was stained for protein using Coomassie Blue (*a*). To the second gel, 10 μl of the above mix was added and after electrophoresis was immunoblotted (*b*). Purified myeloperoxidase (5 μg, lane 9) was loaded to the protein gel and 1 μg to the gel which was immunoblotted. Apparent molecular masses were calculated by comparing the relative mobilities with those of standards (Sigma, SDS-7).

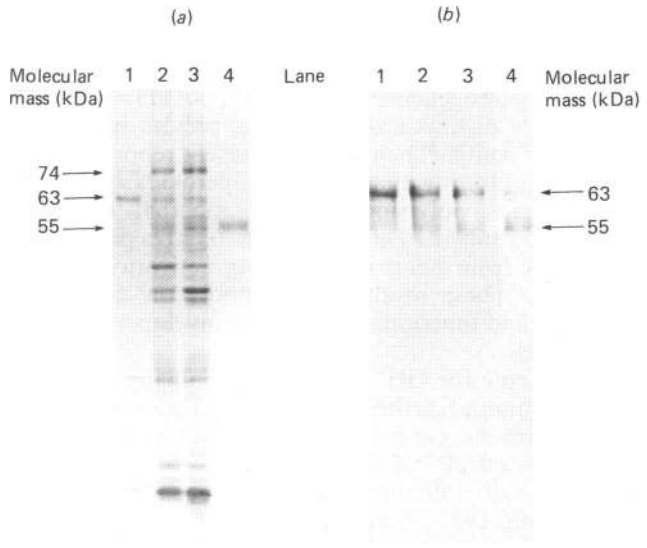

Fig. 2. Protein profiles and immunoblots of neutrophils activated *in vitro*

Neutrophils were purified from the peripheral blood of healthy volunteers and suspended in buffer devoid of bovine serum albumin. Two portions containing 2×10^6 cells were taken and to one of these 1 μM-fMet-Leu-Phe plus 1 μg of cytochalasin B/ml (final concns.) was added. After incubation at 37 °C for 5 min both this and the unstimulated suspension were centrifuged for 2 min at high speed in an MSE Microcentaur and the cell pellets and supernatants retained. The cell pellets were re-suspended in 900 μl of water and proteins were precipitated from these and the cell-free supernatant by adding trichloroacetic acid to 10% followed by incubation at 4 °C for 16 h. The precipitates were then centrifuged and pellets washed five times with ether: after the final wash residual traces of ether were removed by warming to 37 °C. Precipitates were then dissolved in SDS-sample buffer containing β-mercaptoethanol and 25 μl of this mixture loaded for protein staining (*a*) or 10 μl loaded for immunoblotting (*b*). Lane 1, supernatant from activated neutrophil suspensions; 2, cell extract from stimulated neutrophils; 3, cell extract from unstimulated neutrophils; 4, purified myeloperoxidase (5 μg in *a*, 1 μg in *b*). No proteins were detected in cell free supernatants from non-stimulated neutrophils.

Polypeptide profiles and immunoblots of neutrophils and synovial fluid from patients with rheumatoid arthritis

Since it was shown (Fig. 2) that neutrophils which had been activated *in vitro* secrete myeloperoxidase and that this was identified extracellularly, it was then necessary to determine whether this process also occurred during activation *in vivo* in patients with rheumatoid arthritis. Therefore, neutrophils were prepared from the synovial fluid and peripheral blood of a patient with rheumatoid arthritis and the polypeptides were compared together with the synovial fluid itself. The polypeptide profiles of synovial fluid neutrophils and bloodstream neutrophils were remarkably similar except that in the former, polypeptides with apparent molecular masses of 74 and 55 kDa (plus a few other polypeptides) stained less intensely than in bloodstream neutrophils (Fig. 3*a*). Corresponding immunoblots revealed purified myelo-

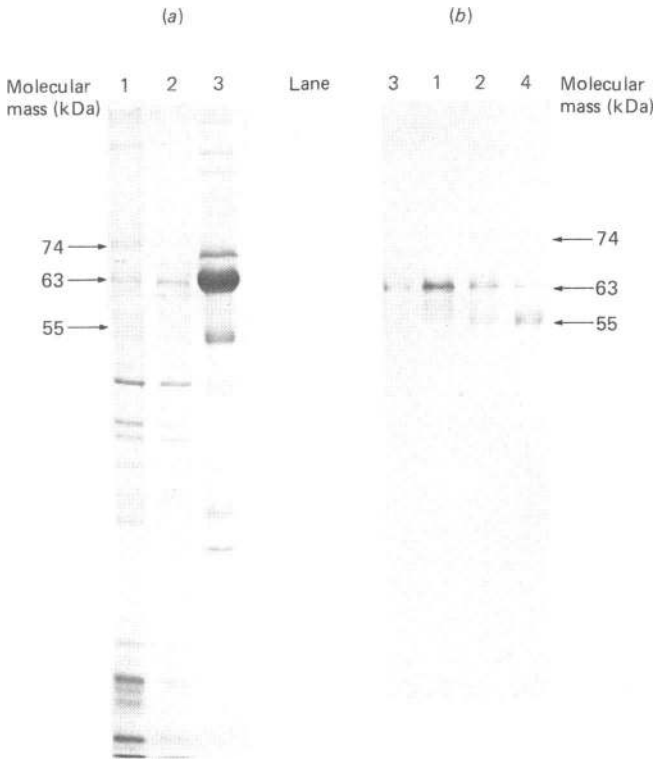

Fig. 3. Protein profiles and immunoblots from neutrophils and synovial fluid from a patient with rheumatoid arthritis

Neutrophils were prepared from venous blood and synovial fluid from a patient with rheumatoid arthritis. Samples of neutrophils (2×10^6 cells), synovial fluid and myeloperoxidase were prepared and loaded as described in the legends to Figs. 1 and 2. (*a*) Protein stained; (*b*) immunoblots. Lane 1, cell extract from bloodstream neutrophils; 2, cell extract from synovial fluid neutrophils; 3, synovial fluid; 4, purified myeloperoxidase.

peroxidase subunits with apparent molecular masses of 55, 63 (and 74) kDa and that the 63 and to a lesser extent the 55 kDa polypeptides were identified in bloodstream neutrophils (Fig. 3*b*). However, in neutrophils isolated from the synovial fluid from the same patient, the 63 kDa subunit stained less intensely than in bloodstream neutrophils and correspondingly this polypeptide was found extracellularly in the synovial fluid (Fig. 3*b*). Essentially the same results were obtained when similar experiments were repeated using samples prepared from a further three patients. In these samples (and in samples from a further three patients) the total myeloperoxidase activity in synovial fluid neutrophils was only 15–20% of that present in corresponding bloodstream neutrophils from the same patients.

DISCUSSION

The results presented here clearly show that synovial fluid from patients with rheumatoid arthritis contain immunologically-detectable, but enzymatically-inactive myeloperoxidase; this protein was quantified by rocket immunoelectrophoresis and its identity as myeloperoxidase was confirmed by comparing immunoblots with those obtained for the purified enzyme. Bloodstream neutrophils may be activated *in vitro* to secrete myeloperoxidase (Edwards *et al.*, 1987*b*) and polypeptides cross-reacting with anti-(myeloperoxidase) antiserum were detected extracellularly (Fig. 2). Comparison of immunoblots of neutrophils isolated from blood and synovial fluid from patients with rheumatoid arthritis showed a reduced level (and activity) of myeloperoxidase in synovial fluid neutrophils (Fig. 3) and correspondingly, immuno-detectable myeloperoxidase released from neutrophils was present in synovial fluid. Thus, these results show that neutrophils in synovial fluid of patients with rheumatoid arthritis have been activated *in vivo* to secrete myeloperoxidase. Since this myeloperoxidase is enzymatically inactive and the enzyme can be oxidatively inactivated *in vitro* (Edwards *et al.*, 1987*b*), it is therefore proposed that in synovial fluid myeloperoxidase has been secreted concomitantly with activation of oxidant generation, although more direct evidence may be required to clarify this latter point.

Much controversy exists in the literature regarding the subunit structure of purified myeloperoxidase, although molecular cloning techniques should help clarify this apparent complexity and confusion (Chang *et al.*, 1986; Johnson *et al.*, 1987; Morishita *et al.*, 1987; Weil *et al.*, 1987). For example, the purified enzyme from human neutrophils or HL-60 cells has been reported to comprise subunits of 55–63 and 10–15 kDa and also a 39 kDa subunit in non- or partially-reduced preparations (Matheson *et al.*, 1981; Olsen & Little, 1984; Olsson *et al.*, 1984; Koeffler *et al.*, 1985; Nauseef, 1986; Nauseef & Malech, 1986). The enzyme is synthesized as a single, large precursor and is then cleaved to yield a number of intermediary polypeptides one of which has a molecular mass of 74 kDa (Koeffler *et al.*, 1985; Akin & Kinkade, 1986). Only a few reports have described preparations containing more than one subunit in the range 55–63 kDa (Pember *et al.*, 1983; Akin & Kinkade, 1986; Miyasaki *et al.*, 1986) but these have been proposed to represent heterogeneities in structure, possibly indicating functionally distinct enzyme species present in separate subcellular compartments. In the present report we show by immunoblotting that our myeloperoxidase preparation contains polypeptides with apparent molecular masses of 55 and 62 kDa and that this 62 kDa polypeptide is secreted from neutrophils during activation *in vitro* or *in vivo*. These results thus support the concept of structural and functional diversity of myeloperoxidase in neutrophils.

Since a role for $OH^{\cdot}$ in the tissue damage associated with rheumatoid arthritis and other forms of inflammatory joint disease has been proposed (Halliwell *et al.*, 1985), the effects of iron chelators to alleviate the symptoms of inflammation have been investigated (Blake *et al.*, 1983; Andrews *et al.*, 1987). However, the activity of myeloperoxidase and its ability to generate reactive oxidants such as HOCl is independent of the level of iron in its environment. Thus, therapeutic regimes designed to restrict available iron levels will limit $OH^{\cdot}$ production, but will not affect oxidant generation by myeloperoxidase. Further work is therefore necessary to evaluate the role of myeloperoxidase products in inflammatory joint disease and it is of note that many anti-arthritis drugs currently in use have been shown to scavenge the myeloperoxidase product, HOCl (Matheson, 1982; Cuperus *et al.*, 1985). Further work is also necessary to correlate the levels of myeloperoxidase released into synovial fluid with the severity of the

disease, in order to fully assess the clinical significance of these observations. Since no specific inhibitor for myeloperoxidase exists (Edwards & Swan, 1986), a supplementary approach to the use of iron chelators in rheumatoid arthritis will now be to search for specific agents which selectively inhibit myeloperoxidase.

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