Activation of the neutrophil myeloperoxidase-H$_2$O$_2$ system by synovial fluid isolated from patients with rheumatoid arthritis

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Abstract
Synovial fluid isolated from 16 patients with rheumatoid arthritis activated luminol dependent chemiluminescence in bloodstream neutrophils, and the maximal activity stimulated varied over a 50-fold range. In contrast, these same fluids only activated a much lower range (two- to threefold) of maximal rates of lucigenin dependent chemiluminescence and cytochrome c reduction, two assays which only measure oxidant secretion which is independent of myeloperoxidase. Over 95% of the luminol dependent chemiluminescence activated by all samples was inhibited by azide (indicating its dependence upon myeloperoxidase), but anti-myeloperoxidase IgG (which specifically inhibits only the extracellular activity of this enzyme) only inhibited the response stimulated by some samples: those fluids which activated the highest luminol dependent chemiluminescence also stimulated the greatest activity of an extracellular myeloperoxidase-H$_2$O$_2$ system. A clear correlation was shown to exist between the activity of myeloperoxidase already present in the fluids (after its secretion from neutrophils in situ within the rheumatoid joint) and the ability of the fluid to activate luminol dependent chemiluminescence. It is concluded, therefore, that all synovial fluid samples tested possess almost equivalent levels of a factor(s) which activated O$_2$/H$_2$O$_2$ secretion and that the variations in the measured activity of the extracellular myeloperoxidase-H$_2$O$_2$ system are dependent upon the level of degranulation which had occurred within the joint.

Polymorphonuclear leucocytes (neutrophils) play a key part in host protection against invading microbial pathogens and may perform other immunoregulatory functions. Because they are present in large numbers in the synovial fluid of patients with rheumatoid arthritis and other types of inflammatory joint disease and also possess a broad spectrum of cytotoxic processes it has been proposed that these cells become inappropriately activated within rheumatoid joints in a mistaken attempt to mount an antimicrobial response. Thus it has been suggested that neutrophil derived cytotoxic products provoke or exacerbate joint damage because naturally occurring host protection systems—for example, oxidant detoxifying enzymes such as catalase and superoxide dismutase, or antiproteinas—may be deficient within the inflamed joint. Indeed, evidence is now accumulating to support this concept that neutrophils have been activated within rheumatoid joints, lending indirect support to this proposal. Central to this hypothesis is the presence within synovial fluid of factor(s) which can activate newly recruited neutrophils, and immune complexes present within these fluids are likely candidates for this role.

When neutrophils become activated in vitro by soluble or particulate stimuli they can generate reactive oxidants either intracellularly or extracellularly. Oxidant secretion may be necessary to kill non-phagocytosable pathogens and may also be important in the activation or deactivation of proinflammatory and anti-inflammatory regulatory components. Intracellular oxidant generation may be restricted to within the phagolysosome in order to target their production towards the ingested pathogen, but it is intriguing to note that intracellular oxidant production can occur in response to soluble stimulants. Although the biological role of this latter production is unknown, it is unlikely to have a direct role in host tissue damage during inappropriate neutrophil activation in inflammation.

We have previously shown that neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis have biochemical characteristics to indicate that they have been both primed and activated in situ within joints. As synovial fluid contains several cytokines these, among other functions, may serve as neutrophil priming agents within the joint to up-regulate the responsiveness of newly recruited bloodstream cells. We have also shown that myeloperoxidase is present in synovial fluid in a form which suggests that it has been secreted from neutrophils concomitant with the secretion of reactive oxidants, and that the activity of this enzyme in synovial fluid neutrophils is lower than in paired bloodstream cells. Thus we proposed that an extracellular myeloperoxidase-H$_2$O$_2$ system plays an important part in tissue damage and perhaps other immunoregulatory processes in this disease. Here we show that synovial fluid from patients with rheumatoid arthritis contains a factor(s) (probably an immune complex) which activates myeloperoxidase dependent and independent reactive oxidant generation in bloodstream neutrophils, and that this factor(s) activates both intracellular and extracellular production. We also show that the amount of extracellular oxidant secretion stimulated by these fluids correlates with the activity of secreted myeloperoxidase contained within these fluids and thus further implicates this enzyme in the pathology of inflammatory joint disease.
Patients and methods

Patients
Sixteen patients attending the rheumatology clinics of the Royal Liverpool Hospital all had classical or definite seropositive rheumatoid arthritis (American Rheumatism Association criteria). Their mean age was 56 years (range 24–80) and disease duration 8–8 years (range 0–3–31). All were receiving non-steroidal anti-inflammatory drugs and no patient had received steroids within the previous six months. Control blood was taken from healthy laboratory personnel.

Preparation of Neutrophils
Neutrophils were prepared from 20 ml heparinised venous blood of healthy laboratory personnel using M-PRM (Flow Laboratories), exactly as described previously.26 After purification they were suspended in a buffer containing (mmol/l): NaCl 120; KCl 4.8; KH₂PO₄ 1.2; CaCl₂ 1.3; MgSO₄ 1.2; HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid) 25 (pH 7.4); 0.1% bovine serum albumin. Cells were counted after a suitable dilution in the above buffer using a Fuchs-Rosenthal haemocytometer slide and used within four hours of preparation. Synovial fluid was collected by aspiration of knee joints and centrifuged at 11 600 g for five minutes: the cell free supernatant was retained and stored in portions at −70°C until use.

Analytical Methods
Neutrophils were suspended in buffer containing either 10 μM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) or 25 μM lucigenin (bis-N-methylacridinium nitrate) at 10⁶ cells/ml in a total volume of 1 ml, and chemiluminescence was measured at 37°C with an LKB Wallac 1250 or 1251 luminometer.

O₂ generation was measured in a continuous assay by monitoring the rate of superoxide dismutase inhibitable reduction of ferricytochrome c.28 The assay, total volume 1 ml, was performed in a Perkin-Elmer Lambda 5 spectrophotometer and contained 75 μM cytochrome c plus 5×10⁵ cells.

Myeloperoxidase activity was measured as the rate of formation of tetraguaiacol from guaiacol.29 Human myeloperoxidase was purified from buffy coats30 and used to raise rabbit anti-(human myeloperoxidase) antiserum, from which a purified IgG fraction was isolated.31 Heat aggregated human gammaglobulin was prepared32 by incubating a purified IgG fraction (isolated from pooled human serum) at 63°C for 60 minutes.

Results

Stimulation of Neutrophil Oxidant Production by Synovial Fluid
Stimulation of reactive oxidant generation by neutrophils after the addition of either soluble or particulate agents can be detected by measurements of luminol dependent or lucigenin dependent chemiluminescence, or by cytochrome c reduction. Cells were suspended, therefore, in buffer containing 10 μM luminol, and chemiluminescence was measured after addition of a range of concentrations of synovial fluids isolated from knee joints of different patients with rheumatoid arthritis. The table shows the maximal luminol dependent chemiluminescence responses obtained with 13 different samples; maximal responses were in the range 0.5–950 cpm and were obtained after the addition of 20% (v/v) synovial fluid to the neutrophil suspension. In the data shown in the table the stimulatory effects of these synovial fluids were all measured on the same preparation of neutrophils isolated from a single donor.

Stimulation of Intracellular and Extracellular Oxidant Generation by Synovial Fluid
Luminol dependent chemiluminescence measures both intracellular and extracellular oxidant production by activated neutrophils.13–15 We have previously shown that these events can be distinguished by a number of approaches. Firstly, stimulation of cells in the presence of anti-(human myeloperoxidase) IgG specifically inhibits the extracellular activity of the myeloperoxidase-H₂O₂ system.13 27 Secondly, measurement of lucigenin dependent chemiluminescence detects extracellular oxidant production,27 which is independent of myeloperoxidase. Thirdly, measurement of cytochrome c reduction, which is superoxide dismutase inhibitable, specifically measures O₂− secretion.28 All these approaches were investigated, therefore, to establish whether synovial fluid activated intracellular or extracellular oxidant production by neutrophils.

Luminol Dependent and Lucigenin Dependent Chemiluminescence
Figure 1 compares the kinetics of luminol dependent and lucigenin dependent chemiluminescence of neutrophils stimulated by three different synovial fluids—those which showed high (a), intermediate (b), and low (c) activity (see the table). After the additions of synovial fluids to the suspensions maximal luminol dependent chemiluminescence was observed after 30–35 minutes and then the rate of oxidant production declined rapidly (fig 1A). When the
same three fluids were used to stimulate luctigenin dependent chemiluminescence (fig 1B) sample (a) again elicited the maximal response, but the maximal rate stimulated was much lower than that seen for lumiophyl dependent chemiluminescence. In contrast, whereas sample (b) activated greater luminol dependent chemiluminescence than sample (c), these two fluids activated identical (barely detectable) lucigenin dependent chemiluminescence. These data suggest that sample (a) only generates significant amounts of extracellular oxidants and that samples (b) and (c) activate the myeloperoxidase-H2O2 system.

EFFECT OF ANTI-(MYELOPEROXIDASE) IgG AND AZIDE ON LUMINOL DEPENDENT CHEMILUMINESCE

We have previously shown that the activities of both the intracellular and extracellular myeloperoxidase-H2O2 system can be detected by incubating cells in the presence of anti-(myeloperoxidase) IgG and azide.13 Two different fluids were chosen for these experiments, which showed either a high (fig 2A) or low activity (fig 2B). In the absence of inhibitor the high activity synovial fluid stimulated luminol dependent chemiluminescence, which reached 50 mV after 35 minutes' incubation (fig 2A). Addition of azide to suspensions before the addition of synovial fluid completely abolished oxidant production, clearly showing the dependence of myeloperoxidase in this oxidant production. In suspensions incubated with anti-(human myeloperoxidase) IgG before stimulation the rate of chemiluminescence was greatly diminished (only 20% of the control response after 35 minutes' incubation) and a maximal value of 20 mV was obtained by one hour. These data thus show that firstly, this high activity synovial fluid activates the myeloperoxidase-H2O2 system (by virtue of the azide sensitivity) and that secondly, much of this activity is extracellular (because it is inhibited by anti-(myeloperoxidase) IgG).

The low activity synovial fluid (fig 2B) activated 15 mV of chemiluminescence, and this also was almost completely inhibited by the addition of azide. After stimulation of neutrophils in the presence of anti-(myeloperoxidase) IgG, slightly greater luminol dependent chemiluminescence was generated. These observations have several implications. Firstly, as with the high activity fluid (fig 2A), the myeloperoxidase-H2O2 system is activated. Secondly, because anti-(myeloperoxidase) IgG did not inhibit this response, much of this activity must be intracellular and hence inaccessible to the IgG. Thirdly, the rates of intracellular, myeloperoxidase-dependent oxidant production (trace 2c) stimulated by both the high and low activity fluids are virtually equivalent. Fourthly, inhibition of the extracellular myeloperoxidase enhances the duration of oxidant production; we have previously shown that this can occur because a product of the myeloperoxidase-H2O2 system inhibits (and
MEASUREMENT OF O₂ SECRETION
Measurement of superoxide dismutase inhabitable, cytochrome c reduction is an estimation of the rates of luminol dependent chemiluminescence and cytochrome c reduction stimulated by different synovial fluid samples. These different samples stimulated widely different rates of luminol dependent chemiluminescence (table), varying over a 30-fold range, but the rates of stimulated cytochrome c reduction were remarkably similar, which with the exception of sample 13, only varied over a 2.3-fold range. (Interestingly, but inexplicably) sample 13 activated the lowest rates of oxidant production of all the samples in both assays used.) Thus these data taken together with those presented in figs 1 and 2 suggest that (a) all these synovial fluids generate remarkably similar levels of O₂ secretion; (b) they all activate an intracellular myeloperoxidase-H₂O₂ system; (c) only some of them activate an extracellular myeloperoxidase-H₂O₂ system, and it is this activity which results in the large variations in luminol dependent chemiluminescence.

CHARACTERISATION OF THE NEUTROPHIL ACTIVATING FACTOR(S) IN SYNOVIAL FLUID
Preliminary experiments showed that the kinetics of luminol dependent chemiluminescence and O₂ secretion stimulated by synovial fluid were distinct from those activated by the commonly used stimuli, fMet-Leu-Phe, phorbol myristate acetate, latex beads, or opsonised zymosan (data not shown). Several reports have suggested that the immune complexes present within rheumatoid joints can serve as neutrophil activating agents. Therefore, we compared the kinetics of luminol dependent chemiluminescence and O₂ secretion activated by synovial fluid and heat aggregated gammaglobulin and found that they were virtually indistinguishable.

This suggested that, at least kinetically, the neutrophil activating factor(s) in synovial fluid resembled an immune complex, and if this were the agent responsible then it should be particulate and readily removed from suspension by centrifugation. Thus the rate and kinetics of synovial fluid activated luminol dependent chemiluminescence were measured by incubating identical neutrophil suspensions with whole synovial fluid and with the particulate (and later resuspended in phosphate buffered saline) and supernatant factors (after centrifugation of fluid at 11 600 g for five minutes). This showed that the particulate fraction possessed over 75% of the activity of whole synovial fluid, confirming that the activating factor(s) was indeed particulate.

MEASUREMENT OF O₂ SECRETION
Measurement of superoxide dismutase inhabitable, cytochrome c reduction is an estimation of the rates of luminol dependent chemiluminescence and cytochrome c reduction stimulated by 13 different synovial fluid samples were therefore compared. These different samples stimulated widely different rates of luminol dependent chemiluminescence (table), varying over a 30-fold range, but the rates of stimulated cytochrome c reduction were remarkably similar, which with the exception of sample 13, only varied over a 2.3-fold range. (Interestingly, but inexplicably) sample 13 activated the lowest rates of oxidant production of all the samples in both assays used.) Thus these data taken together with those presented in figs 1 and 2 suggest that (a) all these synovial fluids generate remarkably similar levels of O₂ secretion; (b) they all activate an intracellular myeloperoxidase-H₂O₂ system; (c) only some of them activate an extracellular myeloperoxidase-H₂O₂ system, and it is this activity which results in the large variations in luminol dependent chemiluminescence.

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discussion
Several lines of independent experimentation have now shown that neutrophils present within the synovial fluid of patients with rheumatoid arthritis have been activated in situ. This evidence includes the comparison of the oxidative metabolism of paired bloodstream and synovial fluid cells and correlation with that activated in vitro, and the detection of patterns of expression of surface antigens which indicate cellular activation. A key feature of the hypothesis that neutrophil derived oxidants and other cytotoxic products have a role in the pathology of inflammatory joint disease is the identification of factor(s) within synovial fluid which can activate newly recruited bloodstream cells. It has been proposed that immune complexes, which are detectable within these fluids and which are known to activate neutrophils in vitro, fulfil this role. In this report we have clearly shown that synovial fluid from patients with rheumatoid arthritis can directly activate bloodstream neutrophils and that this factor(s) may indeed be an immune complex.

Activation of reactive oxidant production by neutrophils is a complex process entailing agonist/receptor occupancy, signal transduction, second messenger production, and the trans-
location and integration of NADPH oxidase components into an active complex.\(^1\) This oxidase may become activated on the plasma membrane, or else on the membrane (derived from the plasma membrane) surrounding the phagolysosome. Concomitant with oxidase activation is the subcellular movement of cytoplasmic granules containing cytotoxic enzymes (including myeloperoxidase), and these granules may fuse either with the intracellular phagocytic vesicle or else with the plasma membrane.\(^34\) This latter process will result in oxidant secretion and release of granule enzymes extracellularly. Therefore, before the role of neutrophil derived oxidants in the pathology of inflammatory joint disease can be established it is essential that firstly, molecular parameters to establish neutrophil activation are defined and secondly, that intracellular and extracellular events are clearly distinguished.

We have previously established methodologies\(^13\) to distinguish between (a) intracellular and extracellular oxidant secretion and (b) myeloperoxidase dependent and independent processes. We have also shown that myeloperoxidase is detected in synovial fluid from patients with rheumatoid arthritis, and that this enzyme is in a form which suggests that it has been cosecreted from neutrophils in parallel with reactive oxidants,\(^25\) indicating that an extracellular myeloperoxidase-H\(_2\)O\(_2\) system may play an important part in tissue damage. Thus we have sought inhibitors of this enzyme (such as salicylhydroxamic acid\(^{35 36}\) and have shown that anti-(myeloperoxidase) IgG can specifically inhibit the extracellular activity of this enzyme.\(^13 27 33\) The data presented here show that factors present in synovial fluid activate the myeloperoxidase-H\(_2\)O\(_2\) system in bloodstream neutrophils, and hence this work provides further evidence in support of the idea that this enzyme has a crucial role in the pathology of inflammatory joint disease.

All synovial fluids examined activated luminol dependent chemiluminescence (as shown in the table), though there was considerable variation (over a 50-fold range) in the magnitude of this response activated by the different fluid samples. This assay measures both intracellular and extracellular activities of the myeloperoxidase-H\(_2\)O\(_2\) system as luminol freely permeates the neutrophil.\(^14\) Lucigenin, on the other hand, does not penetrate neutrophils and the light emission detected is independent of the activity of myeloperoxidase. Hence lucigenin dependent chemiluminescence (and also reduction of cytochrome \(c\)) only measures the rate of oxidant secretion, independently of the amount of degranulation or extracellular myeloperoxidase. It was interesting to note, therefore, that measurements of lucigenin dependent chemiluminescence and cytochrome \(c\) reduction activated by the different synovial fluid samples varied over a much narrower range than the luminol dependent chemiluminescence measurements. Hence these data suggest that the levels of the factor(s) activating the NADPH oxidase to secrete oxidants were fairly similar in the different synovial fluid samples, but that there was a wide variation in the amount of the component responsible for the activation of the extracellular myeloperoxidase-H\(_2\)O\(_2\) system.

Experiments to inhibit myeloperoxidase (or using salicylhydroxamic acid, unpublished observations) showed that over 95% of the luminol dependent chemiluminescence was mediated by the myeloperoxidase-H\(_2\)O\(_2\) system (fig 2). When anti-(myeloperoxidase) IgG was used specifically to inhibit only the extracellular activity of this system, however, only those fluids which activated high rates of luminol dependent chemiluminescence were affected. Interestingly, the residual activity measured after inhibition of the extracellular enzyme was remarkably similar for all synovial fluid samples, further confirming the suggestion that the level of activity of the oxidase activating factor is remarkably similar in all samples tested. These observations clearly indicate that the wide variations in luminol dependent chemiluminescence activated by the different synovial fluid samples correlated with the extracellular activity of myeloperoxidase and not with variations in levels of a factor(s) which activated the NADPH oxidase. Furthermore, the kinetics of oxidant generation were altered in the presence of anti-(myeloperoxidase) IgG: we have previously shown that a product of the myeloperoxidase-H\(_2\)O\(_2\) system inhibits the duration of oxidant release following exposure of the respiratory burst,\(^33 35\) and hence released myeloperoxidase may serve to self regulate oxidant secretion during inflammatory activation.

Two possibilities may account for this wide variation in extracellular activity of the myeloperoxidase-H\(_2\)O\(_2\) system. Firstly, there may be wide variations in the levels of a factor in these synovial fluids which activates degranulation of myeloperoxidase: we could not, however, detect any correlation between the ability of the different synovial fluids to activate degranulation and their ability to activate luminol dependent chemiluminescence (unpublished observations). Second, it was a possibility that functionally active myeloperoxidase was already present in these synovial fluids at different levels (resulting from activation of degranulation of this enzyme within the joint) and that the introduction of this enzyme into the assay was responsible for the variation in extracellular activity. A remarkable correlation was shown to exist between the rates of luminol dependent chemiluminescence and myeloperoxidase activities in the different samples (fig 3). Thus we conclude that (a) a factor(s) is present in synovial fluids from patients with rheumatoid arthritis which activates reactive oxidant secretion in bloodstream neutrophils, and the concentration of this factor is fairly constant in all fluids tested; (b) samples of synovial fluid from different patients contain functionally active myeloperoxidase of varying activity and hence there is considerable variation in the potential of this system to generate HOCl and related oxidants within the joints of these patients. Preliminary experiments indicate that this activating factor may be an immune complex, in accord with earlier suggestions.
It is now apparent that the inflamed joint constitutes a complex system in which amounts of proinflammatory and anti-inflammatory components are likely to be in a dynamic equilibrium. The joint contains cytokines, which may (in addition to other functions) augment the functional responsiveness of neutrophils, macrophages, and also other factors (presumably immune complexes) that can activate reactive oxygen secretion by newly recruited bloodstream phagocytes. Activated neutrophils can themselves secrete cytokines (such as interleukin 1, granulocyte and monocyte colony stimulating factors), and secreted oxidants may damage host tissues or else regulate the activities of proinflammatory and anti-inflammatory components, including phagocytes recruiting/activating factors. The inflamed joint therefore possesses all of the essential features necessary to sustain a self perpetuating inflammatory response and the neutrophil has the potential to play a central part in this inflammatory cascade.

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