Activation of neutrophil reactive-oxidant production by synovial fluid from patients with inflammatory joint disease

Soluble and insoluble immunoglobulin aggregates activate different pathways in primed and unprimed cells

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Cell-free synovial fluid from patients with rheumatoid arthritis stimulated the NADPH oxidase activity in human neutrophils, which reached a peak 15–20 min after addition. Insoluble immunoglobulin aggregates isolated from these fluids activated a similar pattern of oxidase activity. However, when synovial fluid was added to neutrophil suspensions which had been previously exposed to granulocyte-macrophage colony-stimulating factor, the stimulated oxidase activity was biphasic, in that an additional transient activity was observed which reached a peak within 5 min of addition. The additional neutrophil-stimulating activity could not be sedimented by centrifugation at 330 000 *g*-min, and only activated oxidase activity in neutrophils which had previously been primed. The neutrophil-stimulating activity in this soluble fraction was removed by Protein A affinity chromatography, and activity was recovered in eluates from this column. Thus activity in this soluble fraction from synovial fluid is attributed to the presence of soluble immunoglobulin aggregates. Whereas oxidase activity stimulated by the insoluble immunoglobulin aggregates was inhibited by staurosporine (and hence largely dependent on the activity of protein kinase C), the activity stimulated by the soluble immunoglobulin aggregates was staurosporine-insensitive. The soluble immunoglobulin aggregates were present at significantly higher levels in synovial fluids from patients with rheumatoid arthritis compared with those from other joint arthropathies. Thus rheumatoid synovial fluids possess heterogeneous immunoglobulin aggregates which activate neutrophils via distinct molecular pathways. As neutrophils within rheumatoid joints are primed, the soluble immunoglobulin aggregates are likely to be of importance in disease pathology.

INTRODUCTION

The production of a series of reactive oxygen metabolites during the respiratory burst of neutrophils and macrophages is recognized to be a key event for host protection during infections [1–3]. However, in addition to this protective role during pathogen killing, it is also appreciated that inappropriate oxidant production by neutrophils, for example in inflamed tissues or in inflammatory diseases, may result in host tissue damage [4,5]. Thus it is assumed that, in inflammatory joint diseases such as rheumatoid arthritis, neutrophils become attracted from the bloodstream into synovial joints and then become activated to secrete their reactive oxidants and degradative enzymes. Several lines of evidence are now emerging to show that synovial fluid contains oxidatively damaged components [6,7], neutrophil enzymes are present in such synovial fluids [8,9] and neutrophils isolated from these joints possess biochemical properties to indicate that they have been activated *in vivo* [10–14].

Central to the hypothesis that newly recruited bloodstream neutrophils become activated within inflamed joints is the presence within synovial fluids of factors which are capable of inducing oxidant and enzyme secretion from bloodstream cells. We and others have shown that synovial fluids from patients with rheumatoid arthritis contain such neutrophil-activating factors [9,15–18], which are likely to be insoluble immune complexes and/or immunoglobulin aggregates. Hence these immunoglobulin aggregates active luminol-dependent chemiluminescence in bloodstream neutrophils, but, somewhat

surprisingly, these only activate low levels of superoxide dismutase-inhibitable cytochrome *c* reduction [9,18]. Because, unlike luminol, which freely permeates neutrophils [19], cytochrome *c* is a large basic molecule, it only detects O_2^- which is actually secreted from activated neutrophils: the luminol-dependent chemiluminescence activated by immunoglobulin aggregates is thus largely intracellular [20]. Therefore the importance of these insoluble immunoglobulin aggregates in synovial fluid in activating reactive oxidant secretion from neutrophils is questionable.

Neutrophil function *in vivo* is regulated by the activities of cytokines such as γ -interferon, GM-CSF, G-CSF and IL-1 [3,21], which prime the cells into a state of enhanced responsiveness: primed neutrophils have increased levels of expression of several surface receptors, generate greater levels of reactive oxidants upon subsequent stimulation and are more cytotoxic [22–26]. Furthermore, perturbation of cytokine production has been implicated in the pathology of inflammatory disorders, and several cytokines have been detected in the synovial fluid of patients with inflammatory joint disease [27–34]. Hence, neutrophil function within such joints will be regulated by both their exposure to cytokines and the presence of neutrophil-activating factors. The aim of the present work was therefore to determine the kinetics of reactive oxidant generation after activation of bloodstream neutrophils with synovial fluid from patients with inflammatory joint disease and to evaluate the regulatory effects of cytokines on this process. We show that synovial fluids from such patients contain at least two such

Abbreviations used: GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; IL-1, interleukin 1; fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline.

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neutrophil-activating factors. One of these activities is attributed to insoluble immunoglobulin aggregates which activate both primed and unprimed neutrophils with equal efficacy via a protein kinase C-dependent pathway. The second factor, attributed to the presence of soluble immunoglobulin aggregates, only activates [recombinant (r) GM-CSF or γ -interferon] primed neutrophils and results in a rapid, transient, burst of oxidant production via a protein kinase C-independent signalling pathway.

METHODS

Patients

The patient groups studied were those attending the Rheumatology clinics at the Royal Liverpool Hospital. Seropositive rheumatoid arthritis was as defined by the American Rheumatism Association criteria, and other non-rheumatoid joint arthropathies examined were as described in the text.

Preparation of neutrophils

Neutrophils were isolated from 20 ml of heparinized venous blood of healthy volunteers and patients by using Mono Poly-Resolving Medium (Flow Laboratories) as described previously [35]: neutrophils were isolated from the synovial fluid of patients obtained after aspiration of knee joints, as described previously [8]. After purification, neutrophils were washed and suspended in RPMI 1640 medium (Flow Laboratories), and their purity ($> 97\%$) and viability ($> 95\%$) were assessed by Wright's staining and Trypan Blue exclusion respectively. Cells were counted with a Fuchs-Rosenthal haemocytometer slide after a suitable dilution and used within 5 h of preparation.

Preparation of synovial fluid

Synovial fluid was collected by aspiration of joints of patients with knee effusions and collected into heparinized tubes. The fluid was then centrifuged at 1000 *g* for 5 min to remove the cells, which were purified as described above. The cell-free fluid was then either used immediately or else stored in portions at -20°C before fractionation by centrifugation at 11 600 *g* for 2 min: the supernatant was retained, and the pellet was resuspended to an equivalent volume in RPMI.

Neutrophil priming

Suspensions of neutrophils (isolated from the bloodstream of healthy controls or else from the bloodstream and synovial fluid of patients) were incubated for 1 h at 37°C in RPMI 1640 medium at 10^6 cells/ml. Control suspensions contained no further additions, whereas primed suspensions were supplemented with either 50 units of rGM-CSF/ml [36] or 100 units of γ -interferon/ml [25]. After incubation under these conditions, the ability of the cells to generate reactive oxidants was assessed. Similar priming experiments were performed by suspending 10 μl of whole heparinized venous blood in PBS (phosphate-buffered saline: 10 mM-potassium phosphate, 0.9% NaCl, pH 7.4; total volume 1 ml) and incubating for 1 h at 37°C in the presence and absence of 50 units of rGM-CSF/ml before addition of 100 μM -luminol and stimulation of chemiluminescence by synovial fluid or the chemotactic peptide fMet-Leu-Phe (1 μM).

Effects of inhibitors of signal-transduction pathways

The inhibitory effect of the protein kinase C inhibitor staurosporine was tested by incubating neutrophils with 100 nM of this inhibitor for 2 min before stimulation. Extending this preincubation period did not alter the kinetics of subsequent stimulated oxidant production, and these conditions resulted in complete inhibition of the PMA-stimulated NADPH oxidase activity [37].

Protein A affinity chromatography

IgG-containing complexes were removed from synovial-fluid samples by affinity chromatography using Protein A-Sepharose CL-4B [15]. Bound fractions were eluted with 0.1 M-sodium citrate buffer (pH 3.5) and neutralized to pH 7.4 before assay.

Chemiluminescence

Purified neutrophils were suspended in RPMI 1640 medium containing 10 μM -luminol [38] at $(0.5-1) \times 10^6$ cells/ml in a total volume of 1 ml at 37°C . Chemiluminescence was then measured with either a single-channel LKB 1250 luminometer or an LKB 1251 25-tube luminometer. A whole-blood chemiluminescence assay was also utilized [39], by suspending 10 μl of heparinized venous blood in PBS containing 100 μM -luminol. In some instances neutrophils in whole blood were primed as described above before stimulation.

Materials

Protein A-Sepharose CL-4B, luminol, cytochrome *c*, PMA and fMet-Leu-Phe were from Sigma, and γ -interferon and staurosporine were from Boehringer Mannheim. Human rGM-CSF ($\sim 97\%$ purity) was a non-glycosylated peptide from Glaxo and had an activity of ≥ 1.5 m-units/mg of protein in the AML-193 proliferation assay. Dextran (T500) and Ficoll-Paque were from Pharmacia, and Mono-Poly Resolving Medium and RPMI 1640 medium were from Flow Laboratories. All other reagents were of the highest purity available.

RESULTS

Activation of neutrophil oxidant production by synovial fluid

When suspensions of control (unprimed) bloodstream neutrophils were incubated with synovial fluid from a patient with rheumatoid arthritis, a monophasic luminol-dependent chemiluminescence response was obtained which reached a peak value within 15–20 min after addition of fluid (Fig. 1, trace A). Because insoluble immunoglobulin aggregates can be readily removed from synovial fluid by centrifugation [9,18], this fluid was centrifuged at 11 600 *g* for 2 min: after this the supernatant was carefully removed and retained, and the pellet was suspended in a volume of PBS equivalent to that of the original fluid. When the resuspended particulate fraction was added to neutrophil suspensions, a low luminol chemiluminescence response was activated, again reaching a maximum by 15–20 min after addition and of similar magnitude to that observed with whole fluid (Fig. 1, trace C). The neutrophil-stimulating activity in this particulate fraction was completely removed by Protein A affinity chromatography. Thus the neutrophil-stimulating activity observed in Fig. 1 (trace A) was fully recovered in the pellet obtained after centrifugation, and is therefore due to insoluble immunoglobulin aggregates. When the cell-free supernatant was added to control bloodstream neutrophils, no activity above the baseline response was observed (Fig. 1, trace E): thus the cell-free supernatant contained no factors capable of activating reactive oxidant production in unprimed bloodstream neutrophils.

Because several cytokines have been implicated in the pathology of rheumatological disorders and some are detected in synovial fluid (see the Introduction), we then decided to prime the bloodstream neutrophils with GM-CSF before addition of synovial fluid. When whole synovial fluid was added to GM-CSF-treated neutrophils, the luminol-dependent chemiluminescence response obtained was clearly biphasic (Fig. 1, trace B): the magnitude of the response caused by insoluble immunoglobulin aggregates was enhanced, and an additional transient burst of oxidase activity was observed. When the pelleted

Fig. 1. Activation of neutrophils by synovial fluid

Neutrophils (10^6 cells/ml) were isolated from the bloodstream of a healthy volunteer and suspended in RPMI 1640 medium in the absence (○) or presence (●) of 50 units of GM-CSF/ml in a total volume of 0.9 ml. After incubation for 1 h at 37 °C, luminol was added to 10 μ M (final concn.). In (a), 100 μ l of cell-free synovial fluid from a patient with rheumatoid arthritis was added and the chemiluminescence responses were detected with an LKB 1251 luminometer. The same synovial fluid was centrifuged at 11 600 g for 2 min, and the supernatant carefully removed and retained while the pellet was resuspended in a volume of PBS equivalent to that of the original fluid. In (b), the resuspended pellet (100 μ l) was added to primed or unprimed neutrophil suspensions, whereas in (c) the supernatant (100 μ l) was added. Similar results were obtained in at least 20 other experiments using synovial fluid from different patients with rheumatoid arthritis and neutrophils from different donors.

immunoglobulin aggregates were added to primed neutrophils, the peak response was slightly enhanced (Fig. 1, trace D), but the response was monophasic. However, when the cell-free supernatant obtained after centrifugation was added to GM-CSF-treated neutrophils, a rapid burst of oxidase activity reaching a peak at 3 min was observed (Fig. 1, trace F). Thus, synovial fluid contains at least two neutrophil-activating factors. One of these

Fig. 2. Effect of Protein A affinity chromatography on the neutrophil-stimulating activity of synovial fluid

In (a) cell-free synovial fluid was added to neutrophil suspensions and the luminol chemiluminescence generated was measured as described in the Methods section. The fluid was then centrifuged at 11 600 g for 2 min and the supernatant obtained was applied to a Protein A-Sepharose CL-4B column. The unbound eluate was then added to neutrophil suspensions and the luminol chemiluminescence recorded (b). The column was then washed with 0.1 M-sodium citrate buffer, pH 3.5; the eluate was neutralized to pH 7.4 and added to neutrophil suspensions (c). ○, Chemiluminescence responses of unprimed neutrophils; ●, chemiluminescence of neutrophils previously exposed to 50 units of GM-CSF/ml for 1 h.

is readily pelleted by centrifugation, activates a slow chemiluminescence response in either primed or unprimed neutrophils and is presumed to be due to insoluble immunoglobulin aggregates. The second factor only activates primed neutrophils, elicits a rapid, transient, burst of oxidase activity, and could not be sedimented by centrifugation for up to 330 000 g -min.

Protein A affinity chromatography

Fig. 2(a) shows the chemiluminescence traces obtained on addition of whole cell-free synovial fluid to both primed and

Fig. 3. Activation of oxidant production in a whole-blood chemiluminescence assay

Whole heparinized blood (10 μ l) was diluted to 0.9 or 1 ml in PBS and incubated in the absence (○) or presence (●) of 50 units of rGM-CSF/ml for 1 h at 37 °C. After this incubation, luminol was added to 100 μ M (final concn.), and fMet-Leu-Phe (1 μ M) was added in (a) or 10% (final concn., v/v) supernatant from a synovial-fluid sample was added in (b) and the chemiluminescence responses were measured.

Fig. 4. Quantification of the soluble synovial-fluid activity

Whole heparinized blood (from a single donor) was diluted in PBS and incubated for 1 h in the absence (Unprimed; control) or presence (Primed) of 50 units of GM-CSF/ml at 37 °C. Luminol was added to 100 μ M (final concn.), 10% (final concn., v/v) supernatants from different synovial fluids from patients with rheumatoid arthritis were added, and the chemiluminescence responses were measured.

Fig. 5. Activity of the soluble factor in different joint arthropathies

Neutrophils were incubated in the presence of 50 units of GM-CSF/ml for 1 h, and then synovial-fluid supernatants from patients with rheumatoid arthritis or other joint arthropathies were added to 10% (v/v). Chemiluminescence in the presence of 10 μ M-luminol was then recorded.

unprimed neutrophils: this fluid activated a typical monophasic response in unprimed cells and a typical biphasic response in primed cells. This fluid was then fractionated by centrifugation as described in the Methods section, and the supernatant was applied to a Protein A affinity column. Fig. 2(b) shows that the neutrophil-stimulating activity remained bound to the column. When the column was eluted with 0.1 M-sodium citrate (pH 3.5), the factor which stimulated only primed neutrophils was recovered (Fig. 2c) in the eluate. Thus we conclude that this activity is due to the presence of soluble immunoglobulin aggregates in synovial fluid which are only capable of activating primed neutrophils.

Occurrence of the soluble immunoglobulin aggregates in different synovial fluids

It was then necessary to determine the occurrence of the soluble immunoglobulin aggregates in synovial fluid from different patients with rheumatoid arthritis or other joint arthropathies. We also wanted to determine the relative activities of these aggregates in these fluids and therefore decided to assess if a whole-blood chemiluminescence assay could be used for this purpose. When the chemotactic peptide fMet-Leu-Phe was added to 10 μ l of whole blood (diluted to 1 ml in PBS) containing 100 μ M-luminol, very little chemiluminescence above background was observed (Fig. 3a). However, when the whole blood was preincubated with GM-CSF before addition of stimulus, fMet-Leu-Phe stimulated a much greater response. Thus the ability of neutrophils in whole blood to mount a respiratory burst in response to fMet-Leu-Phe requires pre-exposure to priming agents such as GM-CSF.

When the supernatant fraction from a synovial-fluid sample was added to the whole-blood chemiluminescence assay, again, no response above background was observed (Fig. 3b), unless suspensions had been previously exposed to GM-CSF. The kinetics of the responses were identical with those obtained with purified neutrophils. Thus it is possible to use the whole-blood chemiluminescence assay to determine the relative activities of the soluble immunoglobulin aggregates in different synovial fluids.

When supernatants from synovial fluid (10%, v/v) from patients with rheumatoid arthritis were added to suspensions of untreated whole blood, a range of chemiluminescence responses were obtained, but in most cases this was only barely detectable

Fig. 6. Effect of GM-CSF and γ -interferon on neutrophil function

Isolated neutrophils (10^6 /ml) were incubated for 1 h at 37 °C in the absence (○) or presence of 50 units of GM-CSF/mg (●) or 100 units of γ -interferon/ml (■). After addition of luminol to 10 μ M, 10% (final concn., v/v) supernatant of a synovial-fluid sample from a patient with rheumatoid arthritis was added to each suspension and the chemiluminescence responses were measured. Similar results were obtained in five other experiments using synovial fluids and neutrophils from different donors.

Fig. 7. Effect of the soluble immunoglobulin aggregates on synovial-fluid neutrophils

Neutrophils were isolated from the synovial fluid of a patient with rheumatoid arthritis and then incubated for 1 h in the absence (○) or presence (●) of 50 units of GM-CSF/ml. The cell-free synovial fluid was then centrifuged for 2 min at 11 600 g and the supernatant added (10%, v/v) to the neutrophil suspensions (10^6 /ml) in RPMI containing 10 μ M-luminol.

(if at all) above background levels (mean = 0.32 mV, $\pm$ 0.3, n = 18; Fig. 4). However, when the same supernatants were added to suspensions of whole blood which had previously been exposed to GM-CSF, in all cases the chemiluminescence response obtained was enhanced compared with the unprimed response (mean = 3.1 mV, s.d. $\pm$ 2.9; P = 0.005). Thus all synovial-fluid samples from rheumatoid-arthritis patients examined contain soluble neutrophil-activating immunoglobulin aggregates, but the activities of these varies considerably in different patients.

It was then necessary to determine if the soluble neutrophil-activating immunoglobulin aggregates were present in synovial fluid from patients with other joint arthropathies. This was achieved by adding 10% (final concn., v/v) of synovial-fluid supernatants to a suspension of GM-CSF-treated neutrophils (obtained from a single donor) and measuring the relative chemiluminescence responses obtained. A considerable range of activities was observed in synovial fluid from patients with rheumatoid arthritis (Fig. 5, mean = 45 mV, $\pm$ 66 mV; n = 72), but in non-rheumatoid patients (e.g. osteoarthritis, reactive

Fig. 8. Effect of staurosporine on neutrophil activation

Neutrophils were isolated from the blood of a healthy volunteer and incubated for 1 h in the presence of 50 units of GM-CSF/ml. After this period, a further incubation for 2 min in the absence (○) or presence (●) of 100 nM-staurosporine was performed. Luminol chemiluminescence was measured after the addition of 0.1 μ g of PMA/ml (a), insoluble immunoglobulin aggregates (b) or soluble immunoglobulin aggregates (c). These immunoglobulin aggregates were obtained from a single sample after centrifugation of a synovial-fluid sample from a patient with rheumatoid arthritis.

arthritis, psoriatic arthritis, gout) the range of activities observed was significantly (P = 0.003) lower (mean = 5 mV, $\pm$ 7 mV; n = 26). It was not possible to sub-divide non-rheumatoid-patient responses further, because of insufficient numbers of patients with different diseases.

Effect of neutrophil priming *in vitro* and *in vivo*

Several cytokines have been implicated in the pathology of rheumatoid disease, and evidence in the literature suggests that these may also affect neutrophil function *in vivo* [10]. Therefore it was necessary to determine if other cytokines were capable of enabling bloodstream neutrophils to respond to the soluble immunoglobulin aggregates. Fig. 6 shows that γ -interferon had

a similar effect to GM-CSF in up-regulating neutrophil responsiveness to the soluble activating factor.

Neutrophils were then isolated from the synovial fluid of a patient with rheumatoid arthritis, and the effects of the synovial fluid from the same patient on these cells were observed. The neutrophils were then incubated for 1 h in the presence and absence of GM-CSF: the synovial fluid was centrifuged for 2 min at 11 600 *g*, and the supernatant was tested for its ability to activate oxidant production. Fig. 7 shows that the soluble immunoglobulin aggregates in this fluid activated a monophasic chemiluminescence response (peak by 3 min) which was of equal magnitude in synovial-fluid neutrophils incubated *in vitro* in either the presence or the absence of cytokine. These data indicate that synovial-fluid neutrophils have been primed *in vivo* and hence generate reactive oxidants in response to these soluble immunoglobulin aggregates within the joint.

Effect of staurosporine on neutrophil activation

We have previously shown that the NADPH oxidase of neutrophils can be activated via different signal-transduction pathways, which may activate different subcellular pools of oxidase molecules [37]. We therefore examined the effects of the protein kinase C inhibitor staurosporine [40] on neutrophil activation by synovial fluid.

Addition of staurosporine (100 nM) to neutrophil suspensions completely inhibited the chemiluminescence response elicited by PMA (Fig. 8*a*). This treatment also inhibited chemiluminescence activated by insoluble immunoglobulin aggregates by more than 60 % (Fig. 8*b*), indicating that this response is largely dependent on intracellular signal-transduction pathways involving protein kinase C. In contrast, however, staurosporine had little, if any, effect on the oxidase activity stimulated by the soluble immunoglobulin aggregates (Fig. 8*c*). This implies that the signal-transduction pathways responsible for neutrophil activation via the soluble immunoglobulin aggregates are independent of protein kinase C, and hence distinct from those responsible for activation via the insoluble immunoglobulin aggregates.

DISCUSSION

We show here that synovial fluids from patients with rheumatoid arthritis contain at least two factors which can activate reactive-oxidant generation in bloodstream neutrophils. The activity of one of these factors is attributable to insoluble immunoglobulin aggregates which are known to exist in these fluids [9,15–18]: these complexes induce a slow activation in neutrophils, reaching a maximal value by 15–20 min (Fig. 1). The second factor only activates neutrophils which have been primed *in vitro* with agents such as rGM-CSF and γ -interferon or *in vivo* by analogous agents which exist within the joints: this factor induces a rapid transient burst of oxidant production, and its activity is non-sedimentable under the centrifugation conditions employed (maximally 330 000 *g*-min), distinguishing it from the activity attributable to insoluble immunoglobulin aggregates. Its kinetics of activation resemble those observed during activation of neutrophils by soluble agonists such as fMet-Leu-Phe, leukotriene B₄ and C5a [41], rather than the slower activation profiles observed during activation by particulate stimuli such as immunoglobulin aggregates, latex beads and opsonized zymosan [37]. On the basis of our evidence from Protein A affinity chromatography, we conclude that this activity is due to soluble immunoglobulin aggregates present within these fluids.

Considerable evidence now exists in the literature to show that soluble and insoluble immunoglobulin aggregates or immune

complexes activate neutrophils via distinct pathways. Thus, quite different patterns of neutrophil activation have been observed in experiments using either isolated or synthetic complexes of different sizes [42–45], some activating oxidant production and enzyme secretion, others having little or no effect. Soluble and insoluble immunoglobulin aggregates activate neutrophils via occupancy of different Fc γ receptors: Fc γ RIII occupancy primes the cells for subsequent phagocytosis via Fc γ RII [46,47] and activation of neutrophils via Fc γ RIII and Fc γ RII occurs via different signal-transduction pathways [48–50]. Our data presented confirm these ideas because, whereas neutrophil activation via insoluble immunoglobulin aggregates is staurosporine-sensitive (and hence dependent on protein kinase C), that elicited via the soluble aggregates is independent of the activity of this kinase. Furthermore, the kinetics of activation of the oxidase induced by the insoluble and soluble aggregate is quite distinct. We also make the novel finding that the soluble immunoglobulin aggregates only active neutrophils which have previously been primed (either *in vitro* or *in vivo*), whereas that activated by the insoluble aggregates was independent of the past history of the neutrophils.

The soluble immunoglobulin aggregates were present in different amounts in synovial fluids from patients with rheumatoid arthritis and detectable in some other inflammatory joint arthropathies (such as gout, psoriatic arthritis, reactive arthritis). The relative activities of these, on the basis of their ability to stimulate oxidant production in primed neutrophils, were significantly greater, however, in patients with seropositive rheumatoid disease.

There is considerable evidence to indicate that cytokines play an important role in the pathology of inflammatory joint disease. Therefore it is of considerable interest that the soluble immunoglobulin aggregates only activate cytokine-exposed neutrophils. Furthermore, neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis which have been primed *in vivo* [9] were able to generate reactive oxidants in response to these soluble aggregates without further exposure to cytokines *in vitro* (Fig. 7). Hence these data confirm the view that cytokines regulate neutrophil function within rheumatoid joints and suggest that the soluble immunoglobulin aggregates activate neutrophils *in situ* within these joints.

It is known that neutrophils can generate oxidants both intra- and extra-cellularly [10,51] and that these activities are regulated by separate, but inter-related, signal-transduction pathways [37]. The rapid phase of oxidant secretion elicited by fMet-Leu-Phe is independent of the activity of protein kinase C, whereas the later phase of intracellular oxidant production is dependent on the activity of this kinase. It is therefore extremely interesting that, unlike the oxidase activity stimulated by the insoluble immunoglobulin aggregates, that stimulated by the soluble immunoglobulin aggregates was staurosporine-insensitive and hence independent of the activity of protein kinase C. From our model of sequential activation of subcellular pools of oxidase molecules [37], it is predicted that the protein kinase C-independent activity occurs on the plasma membrane, and hence the generated oxidants will be secreted. If the soluble immunoglobulin aggregates do indeed activate reactive-oxidant secretion from neutrophils, then, coupled with the fact that neutrophils within the joint are cytokine-exposed, these are likely to be of considerable importance in the pathology of inflammatory joint disease.

As our previous experiments have shown that the insoluble immunoglobulin aggregates isolated from synovial fluid do not activate reactive oxidant or granule enzyme secretion from unprimed neutrophils [18], it is unlikely that these are of importance in the joint damage mediated by activated neutro-

phils. The possibility should thus be explored that it is in fact the soluble immunoglobulin aggregates which activate the secretion of neutrophil-derived cytotoxic products within the rheumatoid joint, in order to identify the molecular processes responsible for joint destruction.

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