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Blockade of PAF receptors controls interleukin-8 production by regulating the activation of neutrophil CD11/CD18

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Abstract

The production of interleukin-8 by neutrophils in response to particulate stimuli may play a role in the recruitment and activation of further neutrophils in an inflammatory reaction. Here, we have evaluated the sequence of early events leading to interleukin-8 production by phagocytosing neutrophils. Kinetic experiments showed that the phagocytosis of zymosan particles by human neutrophils was rapid in onset. In contrast, interleukin-8 production was more protracted and only detectable 6 h later. Nevertheless, inhibition of phagocytosis with cytochalasins B or D suppressed the late interleukin-8 production. Activation of neutrophils with zymosan failed to enhance CD11/CD18 expression on the neutrophil surface but led to an increase in the expression of an activation-dependent epitope on CD11/CD18. Pretreatment with the platelet-activating factor (PAF) receptor antagonist, UK-74505 (4-(2-chlorophenyl)-1,4-dihydro-3-ethoxycarbonyl-6-methyl-2-[4-(2-methylimidazol[4,5-*c*]pyrid-1-yl)phenyl]-5-[*N*-(2-pyridyl)carbamoyl]pyridine), significantly blocked the increase in the expression of the activation epitope, resulting in inhibition of the phagocytosis of zymosan and interleukin-8 production. In conclusion, the activation of neutrophils with zymosan leads to the activation of PAF receptors and this is followed by activation of CD11/CD18, phagocytosis of zymosan particles and subsequent interleukin-8 release.

Keywords: Neutrophil; Zymosan; Phagocytosis; Interleukin-8; PAF (platelet activating factor); CD11/CD18 activation

1. Introduction

Neutrophils play an essential role in the defense against invading microorganisms (Showell and Williams, 1989). In response to chemical signals generated in the infected tissue, neutrophils migrate to the inflamed site where they recognize, phagocytose and destroy the foreign agent (Showell and Williams, 1989). The importance of neutrophils and hence neutrophil phagocytosis for host defense is highlighted by patients with leukocyte adhesion deficiency I (Anderson and Springer, 1987; Arnaout, 1990). In these patients, deficiency in the expression of the cell adhesion molecule CD18 leads to impaired tissue recruitment of neutrophils and life-threatening recurrent infections (Anderson and Springer, 1987; Arnaout, 1990). During the phagocytic process, neutrophils release various inflammatory mediators which contribute to the local inflammatory response, including lipid mediators (e.g. platelet-activating factor (PAF)), reactive oxygen species, and chemokines, such as interleukin-8 (Smith, 1994; Worthen and Downey, 1996). Under normal circumstances, the invading pathogen is destroyed, the acute inflammatory response is resolved and tissue reorganization occurs. However, persistent and/or uncontrolled activation of neutrophils may lead to tissue injury and inflammatory disease (Henson and Johnston, 1987; Worthen and Downey, 1996).

There has been much interest in the ability of inflammatory cells to produce interleukin-8 as this chemokine appears to play a major role in the pathophysiology of many acute neutrophil-mediated inflammatory diseases (Baggiolini and Loetscher, 2000; Murphy et al., 2000). We have been interested in the stimuli, especially particulate stimuli, capable of activating neutrophils to produce chemokines, such as interleukin-8. Neutrophils produce significant amounts of interleukin-8 when stimulated with zymosan (Au et al., 1994; Bazzoni et al., 1991). Moreover, blockade of the β_2-integrin CD18 or blockade of PAF receptors during the first 2 h following incubation of

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zymosan with neutrophils abolishes interleukin-8 production measured over a 24-h period (Au et al., 1994, 1998). These results demonstrate an important role for both CD18 and PAF in the process of interleukin-8 production. As blockade is only effective during the first 2 h, these observations imply that an early event following the recognition of zymosan by neutrophils triggers intracellular pathways leading to interleukin-8 production. In the present study, we have evaluated the sequence of early events leading to interleukin-8 production by phagocytosing neutrophils. Specifically, we have investigated whether the lipid mediator PAF played a role in the early activation of CD11/CD18, as CD11/CD18 function is a defining step in the phagocytosis of zymosan particles and subsequent interleukin-8 generation by neutrophils.

2. Materials and methods

2.1. Materials

Zymosan A (from *Saccharomyces cerevisae*), bovine serum albumin, sodium azide, polymixin B sulphate, and protamine sulphate were purchased from the Sigma, Poole, UK. Polyethylene glycol 6000 and Hema Gurr stains was from Merck, Poole, UK. Hydroxyethyl starch (Hespan) was from Du Pont Pharmaceutical, Herts, UK. Percoll was from Pharmacia, Milton Keynes, UK. Foetal calf serum, Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS), RPMI 1640 culture medium containing L-glutamine and antibiotic–antimycotic (penicillin, streptomycin, and amphotericin) were from Gibco, Scotland, UK. Goat anti-mouse IgG (immunoglobulins) fluorescein isothiocyanate (FITC) conjugate was from DAKO, Denmark. Donkey anti-goat IgG was from Nordic Immunological Laboratories, Tilberg, The Netherlands. Mustine hydrochloride was from the Boots, Nottingham, UK. $Na^{125}I$ was from Amersham International, Aylesbury, UK.

The following were generous gifts: human recombinant interleukin-8 was from Dr. I. Lindley (Novartis, Austria), anti-CD18 antibody 6.5E and its control antibody MOPC-21 were from Dr. M. Robinson (Celltech, Berkshire, UK), the PAF receptor antagonist UK-74505 (4-(2-chlorophenyl)-1,4-dihydro-3-ethoxycarbonyl-6-methyl-2-[4-(2-methylimidazol[4,5-*c*]pyrid-1-yl)phenyl]-5-[*N*-(2-pyridyl)carbamoyl]pyridine) was from Dr. J. Parry (Pfizer Central Research, Kent, UK), goat anti-human interleukin-8 antiserum was from Dr. H. Showell (Pfizer Central Research, CT, USA), monoclonal antibody 24 was from Dr. N. Hogg (Imperial Cancer Research, London, UK).

2.2. Radioimmunoassay for human interleukin-8

Immunoreactive interleukin-8 concentration in cell-free supernatant were measured using specific human interleukin-8 radioimmunoassay, as described previously (Au et al., 1994, 1998). Samples (100 μl) were mixed with ^{125}I-human interleukin-8 (50 μl; 0.5 ng) and 50 μl of goat anti-human interleukin-8 anti-serum (1:2000 dilution). After an overnight incubation at room temperature, 25 μl of second antibody, donkey anti-goat IgG (1:20) was added to each sample. After a further incubation overnight at room temperature, the competitive reaction was stopped by addition of PBS/azide (1 ml) and immediate centrifugation at $5422 \times g$ for 10 min. Following aspiration of the supernatant, pellets were counted in a gamma-counter.

Interleukin-8 concentration in each sample was determined by reference to a standard curve for the corresponding protein established over a concentration range of 19.5–10,000 pM. An inter-assay sample (2000 pM) was present in all assays to monitor the variation between each individual assay. Non-specific binding ($4.80 \pm 0.61\%$ of total binding, $n = 10$) was determined by incubating the labelled ligand under identical conditions but in the absence of antiserum. All samples were assayed in duplicate.

2.3. Preparation of human neutrophils

Human neutrophils were isolated as described previously (Nourshargh et al., 1992). Briefly, citrated blood was collected from healthy donors and the red blood cells sedimented by incubation with hydroxyethylstarch (Hespan) for 1 h. Neutrophils were separated from the mononuclear cells and the remaining red blood cells by centrifugation through a two-layer discontinuous Percoll gradient (70%/81%; $1207 \times g$; 30 min). Purity of the neutrophil preparation was greater than 98% with contaminating cells being predominantly eosinophils. Cells were washed three times in Ca^{2+}- and Mg^{2+}-free PBS before resuspension in RPMI 1640 medium containing 10% heat-inactivated foetal calf serum.

2.4. Cell culture

Human neutrophils (2×10^6 cells/well) were plated out in 24-well tissue culture plates (Nalgene Nunc, USA) in a final volume of 500 μl/well. Zymosan was boiled, washed, sonicated, and resuspended at 10^9 particles/ml in RPMI 1640. Cells were then stimulated with zymosan (10^7 particles/well) in a controlled environment (95% CO_2; 5% O_2; 37 °C) for up to 24 h. Polymixin B sulphate (10 μM) was routinely added to each sample to inhibit the effects induced by any LPS contamination. Cell-free culture supernatant was obtained by centrifugation of the supernatant at $300 \times g$ for 10 min. Samples (400 μl) were collected and stored at −20 °C for subsequent measurement of interleukin-8 by radioimmunoassay.

In time-course studies, neutrophils were stimulated with zymosan particles and at different time points afterwards, cell-free supernatant was collected for interleukin-8 measurement. In the blocking experiments, cells were pretreated with 6.5E (10 μg/ml) or UK-74505 (10^{-7} M) for

10 min at 37 °C. This was followed by a further 24-h incubation with zymosan (10^7 particles/well). Supernatant was collected and processed as above.

2.5. Phagocytosis assay

Human neutrophils (2×10^6 cells/well) were plated out in 24-well tissue culture plates (Nalgene Nunc) in a final volume of 500 μl/well. Cells were stimulated with zymosan (10^7 particles/well) and at different time points (15 min to 24 h after stimulation), samples were collected and cytospin preparations made (100 μl; 10^5 cells; $300 \times g$ for 5 min). Slides were fixed in methanol for 5 min and stained with Hema Gurr stains. In each sample, the number of zymosan particles ingested per 100 neutrophils was calculated by counting at least 500 cells and taken as an index of phagocytosis. Each experiment was repeated at least three times using cells from different donors. Viability was assessed by evaluating the ability of live neutrophils to exclude Trypan Blue (0.1%).

To examine the effect of different reagents on the phagocytosis of zymosan by neutrophils, cells were pretreated for 10 min with the following drugs: the anti-CD18 antibody, 6.5E (10 μg/ml) or the matched control antibody MOPC-21 or the PAF receptor antagonist, UK-74505 (10^{-7} M) or vehicle control. After the pretreatments, zymosan (10^7 particles/well) was added to the cells in each well and incubated in a controlled environment (5% CO_2; 95% O_2; 37 °C). After 30-min incubation, samples were recovered from the wells, cytospin preparations made and the degree of phagocytosis determined as described above.

2.6. Flow cytometric analysis

Human neutrophils (2×10^6 cells/well) were stimulated with 10^7 zymosan particles at 37 °C for 30 min. At the end of the incubation, 300 μl of the suspension was transferred into a 5-ml polysterene tube and primary antibody added (either monoclonal antibody 24; 1:10 dilution of cell culture supernatant, or 6.5E; 10 μg/ml, or isotype matched control Ab MOPC-21; 10 μg/ml). After incubation at 4 °C for 30 min, excess antibody was removed by washing twice with ice-cold PBS containing 0.2% bovine serum albumin and 0.1% azide. The bound monoclonal antibody was detected using FITC-conjugated goat anti-mouse IgG (1:20; Fab; DAKO). Secondary antibody incubations were carried out for 30 min at 4 °C followed by two further washes in ice-cold PBS buffer. FITC-fluorescence was analysed using a flow cytometer (FACScan, Becton Dickinson & Co.) and the mean fluorescence intensity was used as an index of the binding of 6.5E or monoclonal antibody 24. In some experiments, cells were pretreated with vehicle or UK-74505 (10^{-7} M) for 10 min at 37 °C. After a further 30-min stimulation with zymosan, the binding of monoclonal antibody 24 on the cell surface was measured as described above.

2.7. Statistical analysis

Data were analyzed using a statistical software package (Instat II) and expressed as mean ± S.E.M. of n experiments. Statistical analysis was performed by Student's t-test and a significant difference between groups was considered if P values were < 0.05.

3. Results

3.1. The early phagocytosis of zymosan by neutrophils underlies their late production of interleukin-8

Initial experiments were set to examine the kinetics of the phagocytosis of zymosan particles by human neutrophils. The phagocytosis of zymosan by neutrophils was rapid in onset (30 min; 206 ± 25 particles ingested per 100 neutrophils) reaching a plateau at 2 h (267 ± 12 particles ingested per 100 neutrophils; Fig. 1). There was no further increase in zymosan uptake at 6 (Fig. 1) or 24 h (data not shown). In contrast, significant immunoreactive interleukin-8 was only detected in the supernatant 6 h after stimulation and continued to increase up to 24 h (Fig. 1). No interleukin-8 was detected from unstimulated neutrophils over 24 h (data not shown). Furthermore, we could not measure any significant amount of neutrophil-associated interleukin-8 after activation with zymosan (data not shown). Pretreatment of neutrophils with drugs which disrupt cytoskeletal changes, cytochalasin B or D, effectively suppressed both the phagocytosis of zymosan parti-

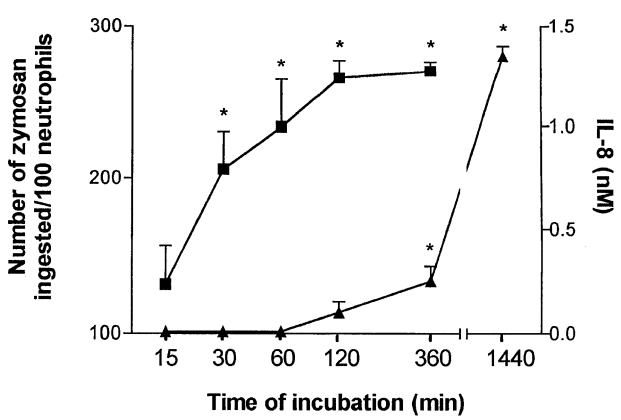

Fig. 1. Time course of the phagocytosis of zymosan particles and interleukin-8 generation by human neutrophils. Human neutrophils (2×10^6 cells/well) were stimulated with zymosan (10^7 particles/well) for various time periods up to 24 h. At the specified times, the number of zymosan particles ingested per 100 neutrophils (closed squares) and the levels of immunoreactive interleukin-8 (closed triangles) were assessed. Results are expressed as the mean ± S.E.M. of $n = 3$. $^*P < 0.01$ when compared to levels in unstimulated cells.

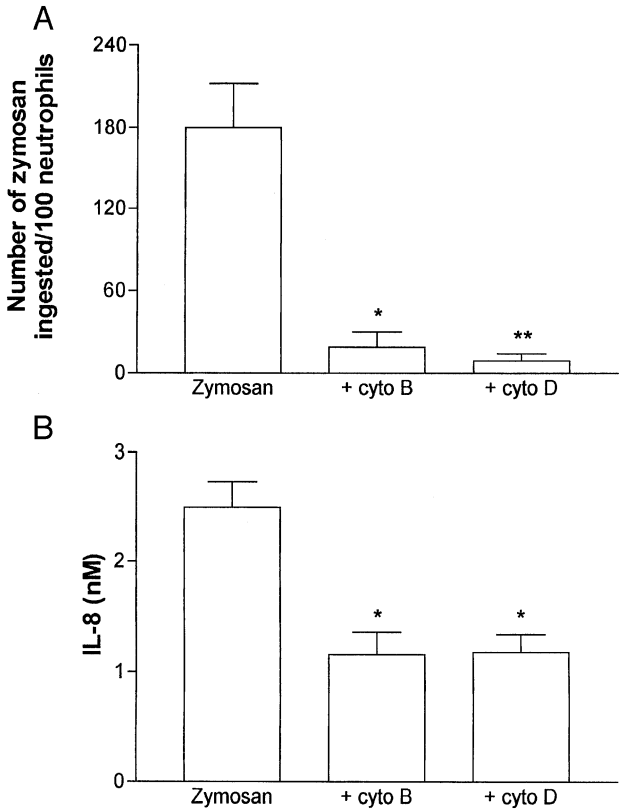

Fig. 2. Effects of agents which disrupt filament formation, cytochalasin B and D, on the phagocytosis of zymosan and interleukin-8 generation by human neutrophils. Neutrophils (2×10^6 cells/well) were pretreated with cytochalasin B (cyto B, 10 μg/ml) or cytochalasin D (cyto D, 10 μg/ml) for 10 min at 37 °C and this was followed by the addition of zymosan (10^7 particles/well). After a further 30 min, the supernatants were removed, cytospins were prepared and the number of zymosan particles ingested per 100 neutrophils (A) assessed. In parallel experiments, immunoreactive interleukin-8 was measured in the 24-h cell-free supernatant (B). Results are expressed as the mean ± S.E.M. of $n=5$. Significant inhibition by cytochalasin B or D is indicated by $^{*}P<0.05$ or $^{**}P<0.01$.

cles (Fig. 2A) and the production of interleukin-8 by neutrophils (Fig. 2B). Not only phagocytosis at 30 min was inhibited by cytochalasins, but also that observed after 6 h (data not shown). None of these compounds had any effect on cell viability over 24 h (data not shown). These results are consistent with our previous suggestion (Au et al., 1994) that an early event (phagocytosis) following the recognition of zymosan particles triggers interleukin-8 production by neutrophils. For all the experiments described below, the phagocytosis of zymosan particles by neutrophils was measured at 30 min and interleukin-8 production was quantified at 24 h.

3.2. Phagocytosis of zymosan particles induces the expression of an activation epitope of CD11/CD18 on neutrophil

The CD18 family of adhesive glycoproteins are important in the recognition of particulate stimuli (Ross et al., 1985; Ezekowitz, et al., 1985), and we have previously shown that anti-CD18 monoclonal antibodies block the production of interleukin-8 by neutrophils activated with zymosan (Au et al., 1994). Anti-CD18 blocked not only interleukin-8 production (zymosan: 1.99 ± 0.49 nM interleukin-8, zymosan + 6.5E (10 μg/ml): 0.17 ± 0.04 nM interleukin-8, $n=6$, $P<0.01$), but also the phagocytosis of zymosan particles by human neutrophils (zymosan: 156 ± 12 particles/100 neutrophils, zymosan + 6.5E: 47 ± 11 particles/100 neutrophils, $n=6$, $P<0.01$). These data suggest that a CD18-dependent interaction is an event which occurs prior to the phagocytosis of zymosan particles by neutrophils. We then examined whether zymosan could modulate the number and/or activation state of CD18 molecules on the neutrophil surface. In unstimulated neutrophils, there was a high basal binding of the anti-CD18 antibody 6.5E in comparison with the non-specific binding of FITC-labelled goat anti-mouse IgG (fluorescence intensity of 742 ± 250 and 7 ± 2, respectively; $n=4$). More-

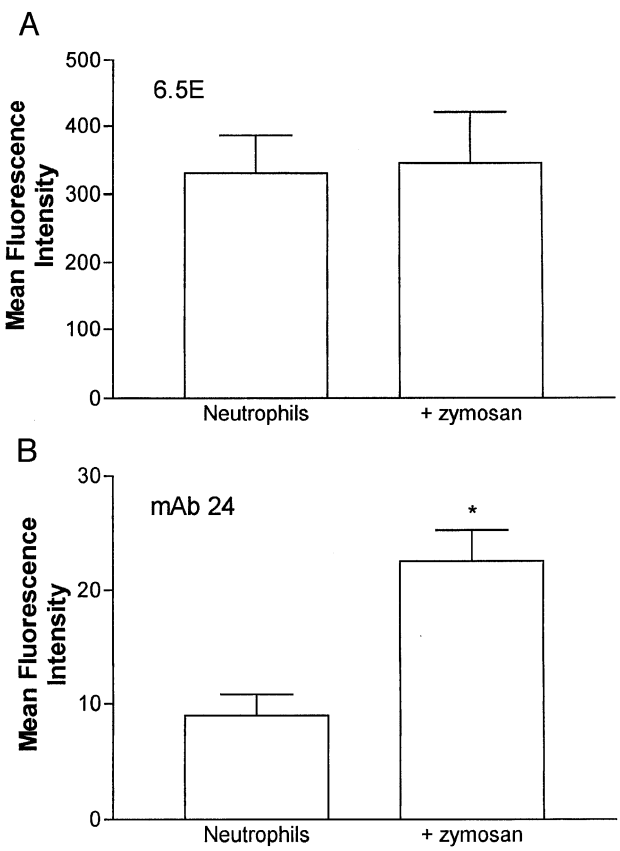

Fig. 3. Effects of zymosan on the number and the state of activation of the integrin CD11/CD18 on the surface of human neutrophils. Cells were incubated with zymosan or medium-control at 37 °C for 30 min. The supernatants were removed and the cells incubated with 6.5E (A) or monoclonal antibody 24 (B) for a further 30 min at 4 °C. Excess antibody was removed by washing the preparation two times and the bound monoclonal antibody detected using FITC-conjugated goat anti-mouse IgG (1:20 dilution). Results are expressed as the mean fluorescence intensity ± S.E.M. of $n=5$. A significant increase in antibody binding above the medium-control is denoted by $^{*}P<0.05$.

over, incubation of neutrophils with zymosan particles did not increase significantly the number of CD18 molecules expressed on the surface of neutrophils (Fig. 3A). In contrast, activation of neutrophils with zymosan markedly enhanced the activation state of CD11/CD18, as assessed by using monoclonal antibody 24 which detects an activation-dependent epitope on CD11/CD18 (Dransfield and Hogg, 1989; Dransfield et al., 1992) (Fig. 3B). There was no significant binding of monoclonal antibody 24 to unstimulated neutrophils compared with the non-specific binding of FITC-labelled goat anti-mouse IgG (fluorescence intensity 9.4 ± 1.2 and 7.0 ± 2.4, respectively; $n = 5$). No significant binding was observed using the isotype matched control antibody MOPC 21 (data not shown). Thus, incubation of neutrophils with zymosan induces the activation but not the increase in the number of CD18 molecules on the neutrophil surface. Activation of CD18 appears to be essential for the phagocytosis of zymosan particles and the production of interleukin-8 by human neutrophils.

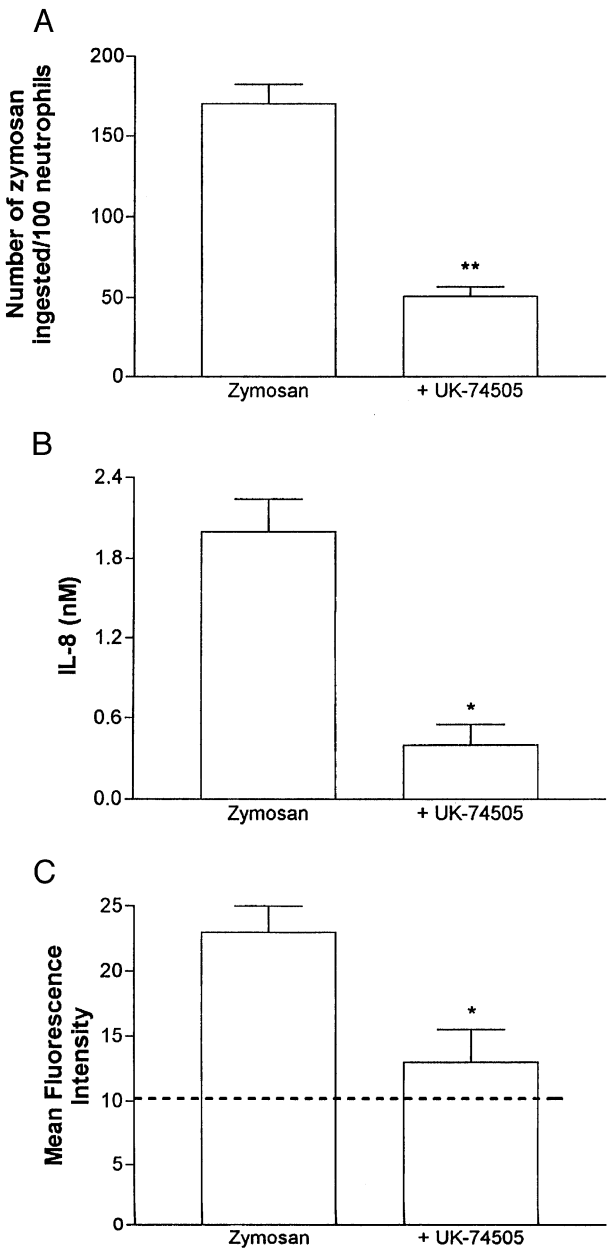

Fig. 4. Effects of a PAF antagonist, UK-74505, on the phagocytosis of zymosan particles, the generation of interleukin-8 and the binding of monoclonal antibody 24 to human neutrophils. Neutrophils were pretreated with UK-74505 (10^{-7} M) or medium-control for 10 min at 37 °C and this was followed by the addition of zymosan (10^7 particles/well). After a further 30 min, the supernatants were removed, cytospins were prepared and the number of zymosan particles ingested per 100 neutrophils assessed (A). In parallel experiments, immunoreactive interleukin-8 was measured in the 24-h cell-free supernatant (B). In some experiments, 30 min after activation, the cells were removed, incubated with monoclonal antibody 24 at 4 °C for 30 min and the bound monoclonal antibody 24 detected using FITC-conjugated goat anti-mouse (C). Results are expressed as the mean ± S.E.M. of 3–5 separate experiments. Significant inhibition by UK-74505 is indicated by $^{*}P < 0.05$ or $^{**}P < 0.01$.

3.3. The expression of a CD18 activation epitope, phagocytosis of zymosan and interleukin-8 production are inhibited by the PAF receptor antagonist UK-74505

The role of endogenous PAF in regulating interleukin-8 production was investigated using the potent and selective PAF receptor antagonist, UK-74505 (Parry et al., 1990; Alabaster et al., 1991) (Fig. 4). In agreement with our previous results in which we also used a distinct PAF receptor antagonist (Au et al., 1994), pretreatment of neutrophils with UK-74505 effectively inhibited the ability of neutrophils to generate interleukin-8 by 70% (Fig. 4B). Furthermore, UK-74505 inhibited phagocytosis of zymosan particles by neutrophils by 68% (Fig. 4A). As CD11/CD18 activation is important for phagocytosis, we examined the role of PAF in the latter process. Significantly, pretreatment of neutrophils with UK-74505 reduced the zymosan-induced increase in the activation state of CD18 by 70% (Fig. 4C). UK-74505 had no effect on the basal levels and activation of state of neutrophil CD11/CD18 (data not shown). These results argue for a central role for endogenous production of PAF in modulating the activation state of CD18 molecules expressed on the surface of zymosan-activated neutrophils.

4. Discussion

Phagocytosis of foreign organisms (e.g. bacteria and fungi) or host tissue debris by neutrophils is an essential part of the local host defense and the restoration of normal tissue function. During phagocytosis, neutrophils can release inflammatory mediators, including interleukin-8, which will contribute to a further influx of inflammatory cells. For example, the production of interleukin-8 following cardiac ischaemia and reperfusion appears to be secondary to a C5a-mediated first wave of migrating neutrophils (Ivey et al., 1995). However, neutrophil influx and

activation in tissue also underlies several important inflammatory diseases, such as rheumatoid arthritis and sepsis (Wagner and Roth, 2000). Thus, the understanding of the mediators and mechanisms involved in neutrophil activation may prove useful in the development of novel anti-inflammatory therapy.

Here, we have designed a series of experiments to investigate the sequence of events leading to interleukin-8 generation after activation of neutrophils with zymosan. We have previously shown that both CD18 and PAF are involved in the generation of interleukin-8 by zymosan-activated neutrophils (Au et al., 1994). Significantly, we showed that inhibition/antagonism of these molecules was only effective during the first hour following activation of neutrophils, suggesting that inhibition of an early event was necessary to observe inhibition of subsequent interleukin-8 production (Au et al., 1994). Initial experiments were then performed to compare the time course profile of phagocytosis of zymosan by neutrophils and the resultant production of interleukin-8. The results clearly established that phagocytosis was an early event occurring within 30 min to 2 h with significant interleukin-8 levels in the supernatant becoming apparent from 6 h onwards. Although these events appeared dissociated in time, disruption of the phagocytic mechanism using cytochalasin B or D (Parod and Brain, 1986; Ting-Beall et al., 1995) effectively inhibited interleukin-8 production implying a link between these processes. As the rate of phagocytosis was maximal during the first 30 min, this time point was chosen in all further experiments evaluating phagocytosis. However, the observation that cytochalasins were more effective at inhibiting phagocytosis (around 90% inhibition) than interleukin-8 production may suggest that either complete inhibition of phagocytosis is necessary for abrogation of interleukin-8 production or that portion of the zymosan-induced interleukin-8 release is independent of the phagocytic process. Nonetheless, our results clearly show that phagocytosis is an essential early step for the production of interleukin-8 by zymosan-activated neutrophils.

Our previous study has shown that pretreatment of neutrophils with an anti-CD18 monoclonal antibody suppressed both interleukin-8 production (Au et al., 1994). Here, we have confirmed these results and shown that the phagocytosis of zymosan particles was also suppressed by anti-CD18 pretreatment. We then investigated whether activation of neutrophils with zymosan induced an alteration in the numbers and/or activation state of CD11/CD18 on the neutrophil surface. There was no elevation in the numbers of CD18 molecules, as assessed by the fluorescence intensity of monoclonal antibody 6.5E binding to neutrophils. In contrast, there was a marked increase in the binding of monoclonal antibody 24, which binds to a common activation-dependent epitope on the α-subunits of CD11a, CD11b and CD11c (Dransfield and Hogg, 1989; Dransfield et al., 1992). These results are consistent with the concept that changes in affinity, rather than an increased expression of surface molecules, are most important for the acute activation of CD11/CD18 (Phillips et al., 1988; Springer 1994; Vedder and Harlam, 1988). Thus, it appears that a CD11/CD18-dependent interaction is an event which occurs prior to the phagocytosis of zymosan particles and that activation, rather than increase in numbers of surface integrin molecules, is the key step leading to phagocytosis and subsequent of interleukin-8 production.

There is much evidence to demonstrate that neutrophils release PAF upon activation with zymosan (Betz and Henson, 1980; Elstad et al., 1994; Sanchez-Crespo et al., 1980). As a PAF receptor antagonist modulated the release of interleukin-8 by modulating an early event following the activation of neutrophils with zymosan (Au et al., 1994), we investigated whether PAF also played a role in modulating phagocytosis and the activation of CD11/CD18. Treatment with UK-74505, a potent and selective PAF receptor antagonist (Alabaster et al., 1991; Parry et al., 1990), effectively blocked not only interleukin-8 generation, but also the phagocytosis of zymosan particles and the zymosan-induced CD11/CD18 activation. These results suggest that the endogenous generation of PAF is an early event which precedes the activation of CD11/CD18.

In conclusion, our experiments suggest that following an initial interaction between neutrophils and zymosan particles, there is a rapid endogenous generation of PAF. The PAF produced acts on PAF receptors (blocked by UK-74505) and leads to activation of CD11/CD18 molecules on the neutrophil surface. The activated CD11/CD18 molecules are essential for the phagocytosis of zymosan particles and subsequent interleukin-8 production. Interestingly, interleukin-8 may itself induce the production of PAF by neutrophils (Bussolino et al., 1992), suggesting that complex inflammatory mediator interaction may occur that will further induce neutrophil activation.

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