Inhibition of kinases impairs neutrophil activation and killing of *Staphylococcus aureus*

Bruno SCHNYDER[1], Paul C. MEUNIER and Bruce D. CAR

*The DuPont Merck Pharmaceutical Company, Safety Assessment Laboratories, Newark, DE 19714, U.S.A.

Intracellular phosphorylations polymorphonuclear neutrophils are mediated by kinases, including mitogen activated-protein (MAP) kinases and phosphatidylinositol 3-kinase. In the present study we demonstrate their effector functions upon both ligation of cell-surface seven-transmembrane-spanning receptors by bacterial peptide formylmethionyl-leucylphenylalanine as well as in the process of destruction of *Staphylococcus aureus*. To regulate neutrophil MAP kinases p38 and p44/42, specifically, we made use of their specific inhibitors 10 μM SK&F 86002 (for p38) and PD 098059 (for activating kinase of p44/42). SK&F 86002 was a potent inhibitor (by 70 %) of induced antimicrobial oxygen-radical generation compared with PD 098059 (by 20 %). SK&F 86002 and PD 098059 inhibited mobilization of a dominant neutrophil adhesion molecule, β_2 integrin, from cytoplasmic granules to the plasma membrane by 40 and 10 % respectively, and the combination of the two drugs resulted in a 90 % effect. The combined effect of both drugs was moderate inhibition of bacterial destruction, despite the fact that neither compound had detectable effect on bactericidal activity if applied individually. Bacterial destruction was also inhibited by wortmannin (0.1 μM), the specific inhibitor of phosphatidylinositol 3-kinase, which had previously been described to target various other activations of the neutrophil, including oxygen-radical generation. Although the relative contribution of p38 and p44/42 MAP kinases varied, the marked effects of the combined inhibition of the kinases revealed their concerted actions to be critical for normal neutrophil function.

INTRODUCTION

Human polymorphonuclear leucocytes (neutrophils) constitute an important part of the initial line of defence against intruding micro-organisms. Leucocytes invade sites of infection through activations enabling initial vascular endothelial interactions, passage through the endothelium and extracellular matrix into surrounding tissues toward micro-organisms. This is followed by phagocytosis and destruction of micro-organisms within the phagolysosome [1,2].

The process whereby neutrophils become activated can occur through events signalled by the seven-transmembrane-spanning receptors and the numerically and functionally most significant membrane adhesion molecule, β_2 integrin. There is evidence that postreceptor signals such as kinase-driven covalent phosphorylation events mediate intracellular signals initiated by ligand binding of cell-surface receptors. Protein-phosphorylating kinase and lipid-phosphorylating kinase have been shown, as revealed by inhibition studies in intact and operational neutrophils, to be of significance for adhesion and respiratory-burst function respectively [3,4]. The lipid-kinase inhibitor wortmannin specifically binds to, and blocks, the activation of phosphatidylinositol (PI) 3-kinase and consequently also blocks activation of multiple downstream elements. Activation of Raf, the mitogen-activated protein (MAP) kinase p44/42 and its activating kinase(s) can thereby be impaired by wortmannin in an indirect fashion [5–9]. Protein kinase C and the p44/42 MAP kinase have been shown, as assessed in cell-free systems, to mediate multiple phosphorylations that facilitate assembly of the neutrophil respiratory NADPH oxidase complex. Therefore the pathway of p44/42 MAP kinase is expected to mediate antimicrobial effector functions. This is partially supported by the fact that inhibition of these protein kinases attenuated the generation of antimicrobial oxygen radicals in intact neutrophils [10–14].

The newly discovered homologues of the p44/42 MAP kinase, for example p38 MAP kinase, are key components that are commonly associated with heat, UV light, osmotic and mechanical stress and proinflammatory cytokine induction in the early host responses to injury and infection [15]. As also shown for p44/42 MAP kinase, homologue p38 is activated by phosphorylation on tyrosine, a dual phosphorylation coupled with threonine phosphorylation, in a unique -Thr-Xaa-Tyr- motif located in an activation loop of the catalytic domain proximal to the binding sites of ATP and the kinase substrates [16–20]. The amino acid Xaa differs for MAP kinases p38 and p44/42, contributing to the district specificity for the substrate of these two kinases [21]. Only one of each MAP kinase can be active in some cell types, while both MAP kinase pathways can operate in other cell types, where they serve opposite or overlapping functions [22,23]. These findings were exploited in transfected immortalized cell lines which showed importance in cell proliferation and differentiation [24,25]. Activation of MAP kinase cascades generally result in the activation of transcription factors which regulate protein synthesis. The limited capacity of neutrophils for protein synthesis suggest that MAP kinase pathways in neutrophils may be involved in the activation of protein-synthesis-independent functions. Respiratory burst and mobilization of intracellular granules to the cell surface are effector functions fully operational within 20 min of inflammatory stimulation. The function of the p38 MAP kinase pathway in neutrophils alone or together with other MAP kinase pathways has been addressed in the present study. Investigations were based on the structural differences between MAP kinases that enabled their individual inhibition.

With foregoing seven-transmembrane-spanning-receptor-induced dual phosphorylation of both p38 and p44/42 MAP kinases in activated neutrophils, their specific roles in agonist-induced respiratory burst, up-regulation of surface β_2 integrin

Abbreviations used: fMLP, formylmethionyl-leucylphenylalanine; PI, phosphatidylinositol; MAP, mitogen-activated protein.

[1] Present address and address for correspondence: Institute of Physiology, rue du Musée 5, 1700 Fribourg, Switzerland.

and in bactericidal activity were presently demonstrated by using the selective inhibitors SK&F 86002 (for p38) and PD 098059 (for activating kinase MEK of p44/42) [26,27]. The results were contrasted with those obtained with the PI 3-kinase inhibitor wortmannin. Incubation of neutrophils with wortmannin significantly impaired their ability to kill phagocytosed *Staph. aureus*.

MATERIALS AND METHODS

Isolation and pretreatment of neutrophils with kinase inhibitors

Human neutrophils from healthy volunteers were isolated from 30 ml of peripheral blood drawn into heparinized Vaccutainers. Density-gradient separation on Neutrophil Isolation Medium (NIM) (Cardinal, Santa Fe, NM, U.S.A.) was performed at 1300 rev./min (340 ***g***) at room temperature for 20 min. The pale-red granulocyte layer was washed with ice-cold physiological NaCl solution buffered with 20 mM sodium phosphate, pH 7.5 (PBS), without Ca^{2+} and Mg^{2+}, centrifuged for 10 min at 1000 rev./min (4 °C), and contaminating erythrocytes were lysed by hypotonic shock in 0.2 % (w/v) NaCl solution for 30 s. Neutrophils of approx. 95 % purity were resuspended in Krebs–Ringer or Hanks buffer (GIBCO, Grand Island, NY, U.S.A.) supplemented with 5.5 mM D-glucose. Cells were preincubated at room temperature with gentle shaking for 30–60 min with indicated concentrations of inhibitors of MAP kinase pathway SK&F 86002 and PD 098059 (0.01–30 μM; synthesized by DuPont Merck Pharma and purchased from Calbiochem, San Diego, CA, U.S.A.) dissolved in DMSO (0.1 %, v/v, in culture). Activity of the inhibitors was tested in specific *in vitro* kinase phosphorylation assays (P. Scherle, personal communication). Cells were also preincubated with wortmannin (0.001–0.1 μM; purchased from Sigma, St. Louis, MO, U.S.A.), genistein (0.1–30 μM; from Calbiochem) or calphostin c (0.01–1 μM; from Calbiochem) dissolved in DMSO, or with vehicle solution alone, and applied to the various assays.

Immunoblot detection of MAP kinases and kinase activity assay

Neutrophils (1×10^6) were stimulated with 10^{-8} M formyl-methionyl-leucylphenylalanine (fMLP) for the indicated periods of time. Cells were quickly pelleted and resuspended or stored frozen in 100 μl of 1 % (v/v) Triton X-100 solution, which was buffered with PBS, supplemented with 1 mM sodium orthovanadate, 1 mM PMSF, 5 mM di-isopropyl fluorophosphate, 10 mM NaF and 5 μg/ml each of pepstatin A, leupeptin and aprotinin. After 15 min lysis at 4 °C, the cell solution was centrifuged at 14000 ***g*** for 5–10 min at 4 °C to pellet the nuclei. Supernatant (20 μl) was separated by SDS/PAGE on a 10–20 %-polyacrylamide gradient, run under reducing conditions, electrophoretically transferred to Nitroblot membranes (Costar, Cambridge, MA, U.S.A.) and immunostained by the enhanced-chemiluminescence detection method (DuPont–NEN, Boston, MA, U.S.A.) as previously described [28]. Rabbit polyclonal antibodies to each MAP kinase recognized the dual-phosphorylation activation site exclusively if phosphorylated (New England Biolabs Inc., Beverly, MA, U.S.A.). Antibodies were raised against peptides deriving from the phosphorylated activation site of human p38 or human p44 MAP kinase sequences. This region is very conserved, and thus the antibody against p44 cross-reacts with its closest homologue, p42. It does, however, not cross-react with p38. Antibodies recognizing MAP kinases p44 or p38 also in the unphosphorylated state served as control detection, in particular, in quiescent cells. These antibodies were raised against peptides deriving from regions other than the activation site.

Activity of MAP kinase-activated protein kinase 2, a major substrate of p38 MAP kinase, was measured as previously described [31]. A highly specific substrate peptide deriving from glycogen synthase N-terminus was utilized to measure incorporation of radiolabelled ATP.

Respiratory burst

Generation of oxygen radicals by neutrophils was measured as the superoxide dismutase-inhibitable reduction of horse ferricytochrome *c* (0.8 mg/ml). Before addition of ferricytochrome *c*, isolated neutrophils were pretreated with inhibitors as described initially. Prewarmed 1×10^6 cells in a 1 ml final volume containing 0.25 % (w/v) BSA (fraction V) were pretreated with 5 μM cytochalasin B for 2 min, and fMLP at 10^{-7} M concentration was used as stimulus for 10 min at 37 °C. The stimulus PMA at 10 ng/ml directly activated intracellular protein kinase C independent of a cell-surface receptor and cytochalasin B addition. Neutrophils were quickly centrifuged at high speed (10000 ***g***), and the absorbance of the supernatant measured in a spectrophotometer at 550 nm, subtracting the absorbance at 490 nm [29]. Control stimulations also contained 10 μg/ml superoxide dismutase as a control for specificity of the reaction of superoxide-radical measurement. Dismutase catalyses the conversion of superoxide radicals into H_2O_2. Any oxygen radical reduced in the presence of dismutase (usually none) was subtracted from the mean measurements recorded as net reduction of ferricytochrome *c* of samples without dismutase. All reagents were purchased from Sigma.

Data were subjected to multivariate analysis of variation in Systat (Systat, Inc. Evabston, IL, U.S.A.).

Flow-cytometric cell-surface analysis of CD11b

Neutrophils (1×10^6), pretreated with kinase inhibitors as described initially, were supplemented with 0.25 % (w/v) albumin and stimulated with 10^{-8} M leukotriene B_4 or 10^{-8} M fMLP for 25 min at 37 °C. After washing cells with ice-cold PBS supplemented with 0.5 % (w/v) BSA and centrifugation at 800 rev./min (140 ***g***) for 7 min, they were stained with fluorochrome FITC-conjugated monoclonal antibody specific for human CD11b (Sigma Immunochemicals, St. Louis, MO, U.S.A.), supplemented with 4 % (v/v) human serum, and rotated for 75 min on ice, protected from light. After washing cells twice, analysis was run immediately using the Cell-Quest Software Program® of the Becton–Dickinson (Mountain View, CA, U.S.A.) flow cytometer. The markers for the monovariant histogram were set based on the negative staining control using isotype-matched control antibodies (FITC-conjugated mouse IgG from Pharmingen, San Diego, CA, U.S.A.).

Bactericidal assay

Gram-positive bacteria (*Staphylococcus aureus*; 35548; Precetrol culture from A.T.C.C., Rockville, MA, U.S.A.) from a single-colony stock grown in nutrient-agar-broth medium (Carr Scarborough Microbiologicals, Inc., Decatur, GA, U.S.A.) were cultured overnight in tryptic-soy-broth medium at 37 °C under rotating conditions, and 3 h before initiation of bactericidal assay, bacteria were diluted 1:20 in 10 ml of prewarmed M63 medium to induce an exponential phase of growth within 2 h while shaking at 37 °C. They were centrifuged for 10 min at 2900 ***g*** and resuspended in 25 °C PBS to a D_{550} of 0.4. Opsonization was carried out by adding 2 vol. of fresh human serum and incubating for 1 h on ice. After refilling with 25 °C

PBS, centrifugation and washing once in 25 °C PBS, the bacteria were resuspended in 5 vol. of neutrophil medium, yielding a final D_{550} of 0.02 in the subsequent bactericidal assay; 750 μl of neutrophils (2.5×10^6 cells/ml) and 250 μl of bacteria solution were mixed to yield a final bacteria/neutrophils ratio of 5:1. Co-culture occurred for 180 min in a volume of 20 μl in 96 flat-bottom well plates on a rotating platform at 150 rev./min at 37 °C. Neutrophils were lysed for a short time in ice-cold water, and *Staph. aureus*, following an appropriate dilution of 1:1600 in 25 °C sterilized water, was immediately streaked in triplicate on nutrient-agar plates (Carr Scarborough Microbiologicals) and cultured at 37 °C overnight. The percentage of colony-forming units was calculated relative to bacteria cultured in vehicle solution without neutrophils during the bactericidal assay.

RESULTS

Effect of inhibitors of MAP kinase pathway on fMLP-induced respiratory burst

The MAP kinase inhibitor SK&F 86002 (for p38) inhibited generation of oxygen radicals by 70 % in neutrophils treated with 10^{-7} M fMLP (Figure 1a). This effect was partial at 1 μM and maximal at 10 μM SK&F 86002. MEK inhibitor PD 098059 (for p44/42 pathway) (10 μM) resulted in an overlap of effector function targeting with SK&F 86002, but inhibited to a minor extent (20 %) at maximal effective concentration (Figure 1a). PD 098059 augmented the effect of SK&F 86002 (Figure 1a). Genistein, the natural inhibitor of a broad spectrum of tyrosine kinases, including MAP kinase kinases, reduced maximal respiratory burst by 80 % (Figure 1b). Results obtained with synthetic inhibitors of the MAP kinase pathway were then compared with those obtained with the natural inhibitors wortmannin and calphostin c, the latter targeting protein kinase C. Both wortmannin (0.1 μM) and calphostin c (0.1 μM) completely eliminated oxidase activity (Figure 1b). Complete inhibition by calphostin c was even observed when the respiratory burst was induced by direct activation of intracellular protein kinase C with 10 ng/ml of PMA in a receptor-independent fashion (Figure 1c). PMA-induced generation of oxygen radicals was not, however, modified by SK&F 86002, PD 098059, a combination of both, or by wortmannin, indicating that these three compounds were specifically able to target the receptor-signalled activation of NADPH oxidase.

Inhibitions of mobilization of functionally significant receptors to the cell surface

SK&F 86002 at 10 μM decreased the leukotriene B_4-induced fluorescence signal of β_2 integrin antigen at the cell surface by 48 % (Figure 2a), as assessed by immuno-flow-cytometric detection of the CD11b chain of the integrin. fMLP-induced signal was decreased by 39 % (Figure 2b). PD 098059 decreased CD11b up-regulations by 10 % (Figure 2). These effects were partial at 1 μM drug concentrations. Although maximal effect was reached at 10–30 μM, a minor additional effect was occasionally observed at 100 μM (results not shown). Co-incubation of SK&F 86002 and PD 098059 (10 μM), resulted in $\geqslant 90$ % inhibition (Figure 2). A similar synergistic profile of inhibition was observed for mobilization of CD18 chain of the β_2 integrin heterodimer (results not shown). Inhibitory effects were still present after stimulation for 2.5 h (results not shown). Wortmannin only showed a minimal effect on agonist-induced up-regulation of β_2 integrin (Figure 2). These findings underscore the significance of MAP kinase pathway in such cytoskeletal processes. The up-regulation of β_2 integrin in neutrophils has previously been postulated to be translocation to, and incorporation of secretory vesicles into, the plasma membrane rather than neosynthesis of receptor proteins [30].

Effects of wortmannin and the inhibitors of MAP kinase pathway on neutrophil bactericidal activity

In a bactericidal assay, destruction of *Staph. aureus* by neutrophils occurred after 30–60 min (results not shown) and was

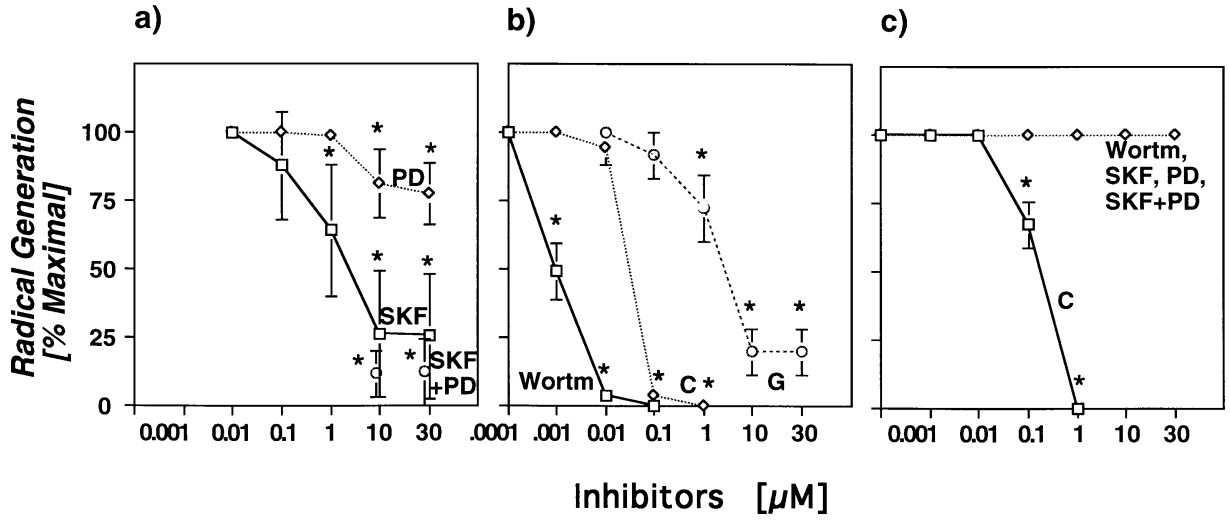

Figure 1 Effect of inhibitors of MAP kinase pathways on respiratory burst

Cells were incubated with PD 098059 (PD), SK&F 86002 (SKF), both inhibitors combined (PD + SKF), inhibitor of broad-spectrum tyrosine kinases genistein (G), inhibitor of protein kinase C calphostin c (C), wortmannin (Wortm), and then in the presence of inhibitors stimulated for 10 min with (**a**,**b**) 10^{-7} M fMLP or (**c**) with 10 ng/ml PMA. Maximal activation without inhibitor pretreatment is defined as 100% (accounts for 9.6 ± 2.1 nmol of O^{2-}/5 min per 10^6 cells). The data presented are means for at least three independent experiments. Error bars represent S.D. Asterisks indicate a statistically significant difference ($P < 0.05$) compared with the positive control.

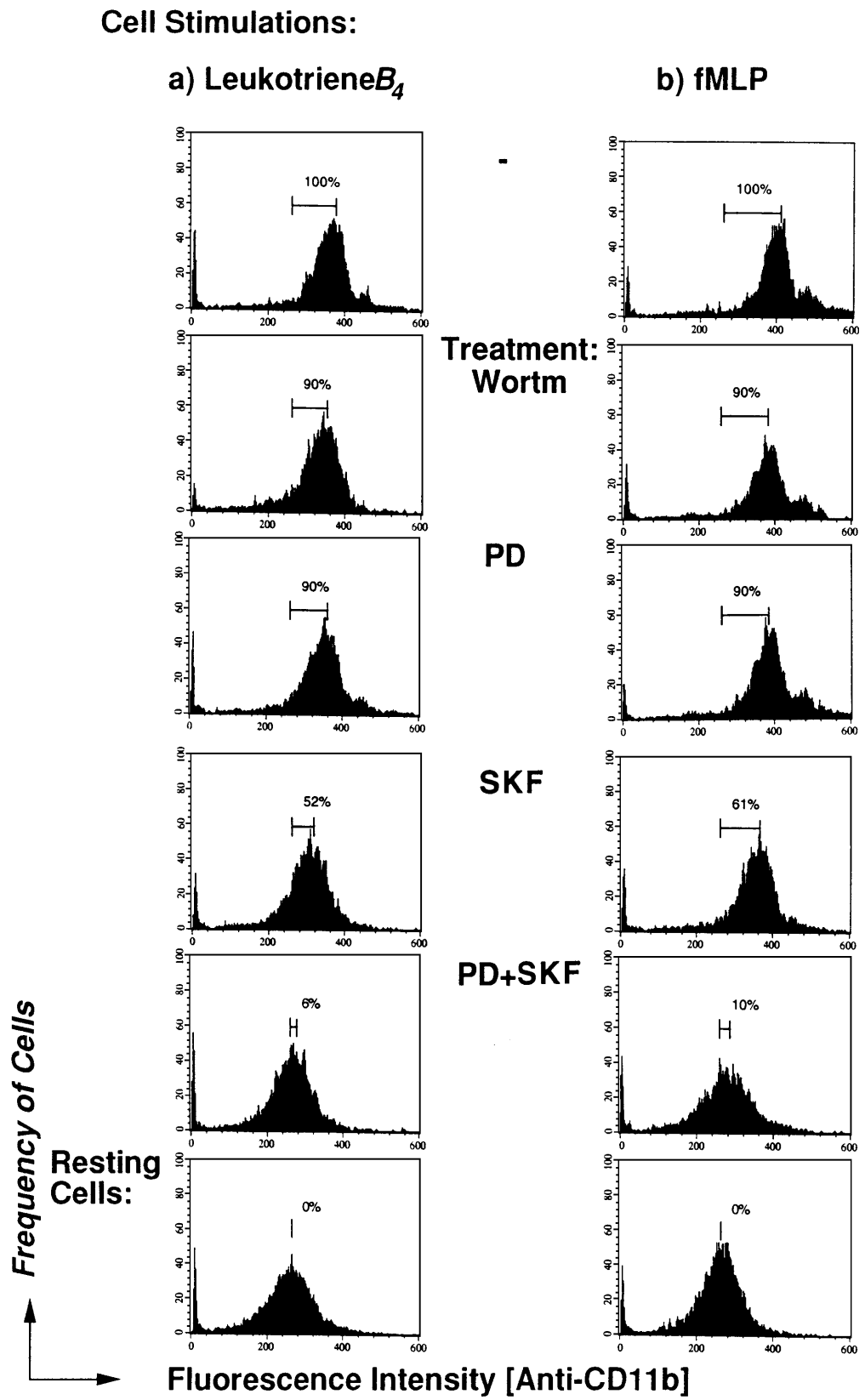

Figure 2 Flow-cytometric analysis of β_2 integrin (CD11b) density on neutrophils

After incubation with the vehicle (—), 10 μM PD 098059 (PD), 10 μM SK&F 86002 (SKF), both inhibitors combined (PD + SKF) or 0.1 μM wortmannin (Wortm), cells were stimulated for 25 min with (**a**) 10^{-8} M leukotriene B_4 or (**b**) 10^{-8} M fMLP. The mean immunofluorescence intensity, given on the abscissa of the flow cytograph, of unstimulated (resting) cells was subtracted from the fluorescence of stimulated cells. The maximal difference represents 100% up-regulation of the antigen, as indicated in the margin drawn between respective peak values. Results shown are representative of five independent experiments.

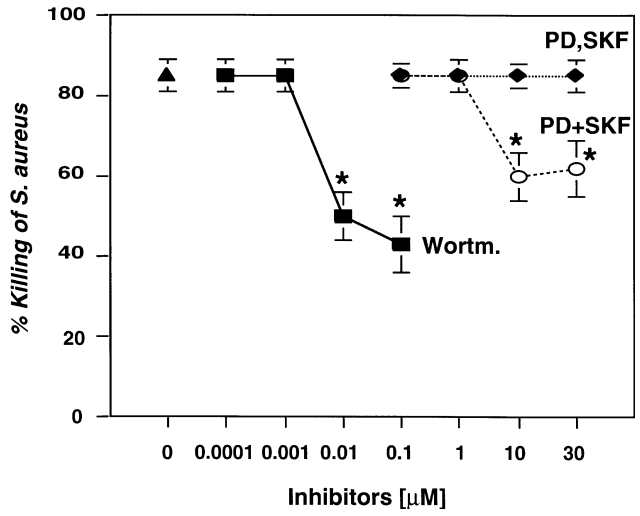

Figure 3 Bactericidal activity of neutrophils was determined following co-culturing of opsonized *Staph. aureus* with neutrophils

After 180 min incubation, surviving *Staph. aureus* were plated on nutrient agar and number of colonies grown counted. The degree of destruction was determined from the difference between bacterial colony counts in the presence and absence of co-cultured neutrophils and was expressed as percentage killing on the ordinate. Reduced destruction was detected when neutrophils were pretreated with a combination of the inhibitors, PD 098059 and SK&F 86002 (SKF + PD) or with wortmannin (Wortm) in lieu of vehicle solution (shown as '0 μM'). The presence of these inhibitor combinations did not impair bacterial proliferation *per se* (results not shown). Results are means ($\pm$S.D.) for at least three independent experiments. Asterisks indicate a statistically significant difference ($P < 0.05$) compared with the positive control.

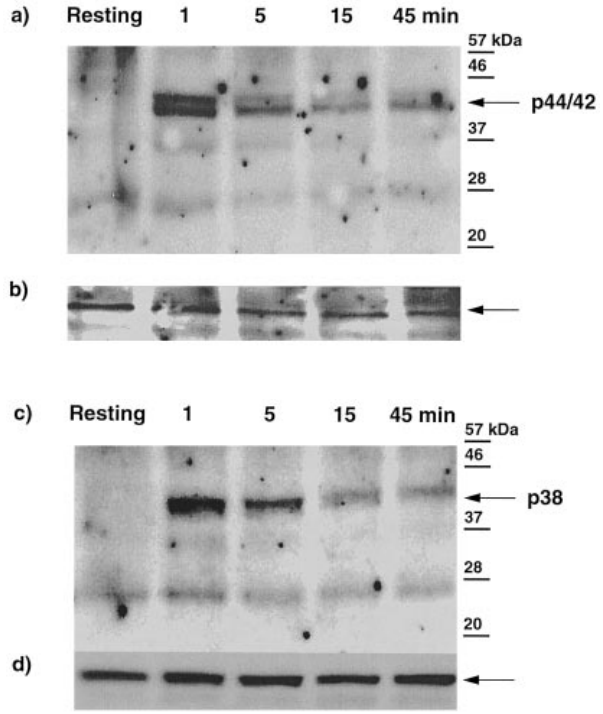

Figure 4 MAP kinases p38 and p44/42 were detected in neutrophils following the electrophoretic separation of cell lysates (SDS/PAGE), electrotransfer and immunostaining

In (**a**) and (**c**), immunodetection of, exclusively, the phosphorylated forms of MAP kinase after stimulation of neutrophils with 10^{-8} M fMLP for the indicated periods of time (min) is shown. In (**b**) and (**d**), immunodetection with antibodies also recognizing unphosphorylated forms were used as controls and revealed the presence of the respective, though unphosphorylated, MAP kinases in lysates of non-stimulated (resting) neutrophils. Induced phosphorylation was also observed when neutrophils were stimulated with 10^{-8} M leukotriene B_4 (results not shown). Molecular mass is indicated to the right of the autoradiograph. Data shown are representative of five independent experiments.

maximal at 180 min co-culture (Figure 3). *Staph. aureus* surviving the bactericidal assay were plated on nutrient agar and number of colonies grown counted. Count differences to reference assays in the absence of neutrophils were expressed as percentage killing on the ordinate of Figure 3. Conditions were optimized to enable maximal bactericidal activity (85% killing) on the one hand. On the other hand, this setting including bacteria opsonization, followed by withdrawal of the serum before the assay, and a high bacteria/neutrophil ratio of 5:1 in the co-culture permitted the sensitive evaluation of effects of various kinase-pathway inhibitors. Although SK&F 86002 and PD 098059 did not separately impair bactericidal activity, when neutrophils were cultured in the presence of both compounds, 60% inhibition of bacterial killing was observed compared with 85% bacterial killing in controls (Figure 3). Wortmannin reduced bacterial killing from 85 to 45% (Figure 3). None of the inhibitors exhibited a direct antibiotic action or promoted bacterial growth in cultures in the absence of neutrophils.

Analysis of the distinct neutrophil p38 and p44/42 MAP kinases

Maximum phosphorylation of 42–44 kDa MAP kinase p44/42 (Figure 4a), in parallel with phosphorylation at the 40 kDa kinase p38 (Figure 4c), occurred within 1 min after 10^{-8} M fMLP stimulation, declining thereafter. Susceptibility of such kinase-mediated phosphorylation to specifically designed MAP kinase inhibitors has previously been described in various cell types. We confirm such inhibitor-susceptible events, specifically endogenous, as phosphorylation at the unique dual-phosphorylation sites in the p44/42 MAP kinase pathway of the neutrophil. Incubation of neutrophils with PD 098059 at the maximal effective concentration of 10 μM blocked induction of phosphorylation of p44/42 (Figure 5a). Also wortmannin (0.1 μM) blocked phosphorylation of p44/42 (Figure 5a).

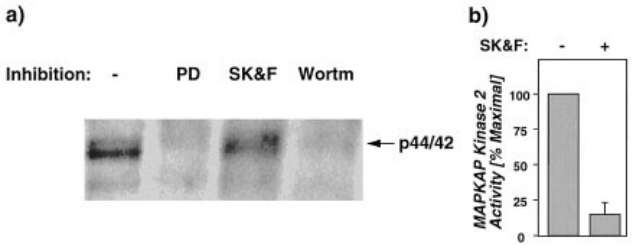

Figure 5 Effect of specific kinase inhibitors on MAP kinases *per se*

Neutrophils were treated with vehicle (—), 10 μM PD 098059 (PD), 10 μM SK&F 86002 (SK&F) or 0.1 μM wortmannin (Wortm) before stimulation with 10^{-8} M fMLP for 3 min. In (**a**), kinase-mediated phosphorylation at the activation site of p44/42 in the lysates was immunoblot-detected as described in Figure 4. The autoradiograph shown is representative of five independent experiments. In (**b**), activity of p38 MAP kinase is indicated by measuring the kinase activity of its substrate MAPKAP kinase 2 in the presence or absence of 10 μM SK&F 86002 in the lysate. '100% specific activity', given on the ordinate, was determined from the difference between stimulated and non-stimulated cells.

Phosphorylation of p38 was unaffected by either compound specifically targeting MAP kinase pathways, except by wortmannin as found occasionally and to a moderate extent of reduction (results not shown). Results of previous studies have shown that binding of the pyridinyl imidazoles of the SK&F 86002 type to the ATP-binding site (Tyr-175) of both activated and non-activated p38 blocks substrate recognition by p38. This drug binding does not modify p38 phosphorylation by MAP-kinase-activating kinases [16,26]. We and others confirmed this (results not shown) [14]. SK&F 86002 blocked fMLP-induced kinase activity of p38 as assessed by measuring activity of its downstream substrate, MAPKAP kinase 2 (Figure 5b), as has also recently been tested by others [32].

DISCUSSION

Neutrophils comprise the initial line of defence against intruding bacterial infections. A model of killing of *Staph. aureus* by neutrophils was established in a co-culture setting. It serves as investigative-toxicology model to test drugs for their effect on normal human immune defence. Drugs of the p44/42 MAP kinase pathway have not yet been tested in inflammatory *in vivo* models. They 'classically' impair cell mitogen effects. The present study demonstrates partial impairment of the bactericidal activity. A combination of two compounds, SK&F 86002 and PD 098059, blocked MAP kinase pathways through p38 and p44/42 respectively. Simultaneous application achieved an effect on the bacterial killing. Effect of simultaneous, in contrast with ineffective individual application of the compounds is possibly enabled by avoiding redundancy of the separable pathways that converge distal to the MAP kinase step on overlapping effector function(s).

The specific inhibitor of PI 3-kinase, wortmannin, has not been tested in inflammatory *in vivo* models. An anti-inflammatory effect would, however, be expected, based on findings obtained with neutrophils. In the more complex inflammatory neutrophil assay of the present study, application of wortmannin clearly impaired bactericidal activity.

Respiratory burst induced by the bacterially derived tripeptide fMLP has previously been linked with the facilitation of destruction of *Staph. aureus* [33,34]. If humans or mice are deficient in the molecular components of the NADPH oxidase complex, the microbicidal activity is impaired [35]. We showed in the present study that the potent effect on radical generation by PI 3-kinase inhibition likely attenuated bacterial killing. Also MAP kinases had an impact on both respiratory burst and killing. fMLP and inflammation-induced, host-derived leukotriene B_4 altered expression of β_2 integrin (CD11b) on the cell surface. Up-regulation of β_2 integrin would seem to be involved in enhancement, rather than initiation, of neutrophil operations such as, for example, respiratory burst [36]. Complete absence of β_2 integrin impaired antimicrobial activity *in vivo* [37]. β_2 integrin has furthermore been shown to operate as an integrin receptor of the serum complement component C3b [37,38]. In fact, *Staph. aureus* in the present study showed dependency on opsonization by serum before it could be destroyed by neutrophils. The induced inhibition of neutrophil MAP kinases in the present study was accompanied by effects on both β_2 integrin up-regulation and bacterial destruction. The neutrophil MAP kinases, in particular, regulated antimicrobial signals through a partially concerted action with PI 3-kinase and a new characteristic mechanism that governs mobilization of granules of β_2 integrin to the cell membrane as well.

Although the natural metabolite wortmannin caused haemorrhagic effects *in vivo* [39], an extended body of studies has employed wortmannin in functional assays. Characterization of synthetic inhibitors of PI 3-kinase pathway are warranted. In the present study, wortmannin served as control inhibitor to elucidate the role of PI 3-kinase in bactericidal activity. Certain processes in endocytosis, as shown in non-phagocytic epithelial cells and macrophages, were impaired by wortmannin [40,41]. Such an inhibitory effect was of little relevance during the process of neutrophils engulfing opsonized *Staph. aureus*, as we assessed microscopically (results not shown) in the present study. Effective bacterial killing started after the initial 20–30 min (the micro-organism uptake) of the assay, and a decrease in killing in the presence of wortmannin might have derived from impaired processes during destruction of the micro-organisms. Protease-granule release in neutrophils has convincingly been shown to be blocked by wortmannin [4,42]. The importance of this process has now been tested in the bactericidal assay. The assay was normal upon addition of a specific inhibitor of neutrophil elastase and proteinase 3 [43] (results not shown). Lysosomal proteolytic degradation of bacteria follows the killing of bacteria, which appears principally to be determined by oxidative mechanisms. The preceding step in microbial destruction, namely neutrophil migration to bacteria, has previously been shown not to be blocked by wortmannin [44]. Recruitment has been mimicked in an assay of neutrophil migration directed through synthetic porous membranes toward a gradient of bacterial peptide fMLP. When chemotoxis was modelled in this system with the host-derived chemoattractant, interleukin-8, wortmannin inhibited the migration of neutrophils [45]. The results obtained with the different, somewhat artificial chemotaxis systems in use are, however, not uniform between groups. Conclusive results based on chemotaxis systems with specific kinase inhibitors, therefore, await further refinement [46] or *in vivo* refinements with well-tolerated and specific kinase inhibitors. Such *in vitro* refinements include addition of physiologically relevant protein matrices or cellular barriers to transmigration.

The synthetic MAP kinase inhibitor SK&F 86002 is not toxic if injected into mice [47]. Doses given *in vivo* decreased collagen-induced arthritis and chemically induced peritonitis in mice. Inflammatory functions of vascular endothelial cells, lymphocytes and macrophages are also modified by the agents inhibiting the MAP kinase pathways used in the present study [47,48]. Collectively, these cell types orchestrate many acute and chronic inflammatory events, suggesting modulation of MAP kinase pathways to be of potential therapeutic use in mitigating inflammatory diseases.

We gratefully thank our colleagues S. I. Carter, C. Clanton, M. Favata, D. Wescott, M. Lorenz, Dr. J. Bogdan Jr. and Dr. S. Schnyder-Candrian for phlebotomy and helpful discussions. We are grateful to Dr. H. Cromwell's presubmission editorial help, and Dr. P. Scherle and Dr. C. Huang for contributing results prior to publication. This work was supported by the DuPont Merck Pharmaceutical Company's postdoctoral programme.

REFERENCES

1 Schnyder, B., Lugli, S., Feng, N., Etter, H., Lutz, R. A., Ryffel, B., Sugamura, K., Wunderli-Allenspach, H. and Moser, R. (1996) Blood **87**, 4286–4295
2 Luscinskas, F. W., Kansas, G. S., Ding, H., Pizcueta, P., Schleiffenbaum, D. E., Tedder, T. F. and Gimbrone Jr., M. A. (1994) J. Cell Biol. **125**, 1417–1427
3 Fuortes, M., Jin, W. W. and Nathan, C. (1993) J. Cell Biol. **120**, 777–784
4 Dewald, B., Thelen, M. and Baggiolini, M. (1988) J. Biol. Chem. **263**, 16179–16184
5 Ward, S., June, C. H. and Olive, D. (1996) Immunol. Today **17**, 508–510
6 Knall, C., Young, S., Nick, J. A., Buhl, A. M., Worthen, G. S. and Johnson, G. L. (1996) J. Biol. Chem. **271**, 2832–2838
7 Downey, G. P., Butler, J. R., Brumell, J., Borregaard, N., Kjeldsen, L., Sue-A-Quan, A. K. and Grinstein, S. (1996) J. Biol. Chem. **271**, 21005–22111
8 Edwards, S. W. and Watson, F. (1995) Immunol. Today **16**, 508–510

9 Thelen, M., Wymann, M. P. and Langen, H. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 4960–4964
10 Grinstein, S. and Furuya, W. (1992) J. Biol. Chem. **267**, 18122–18125
11 Torres, M., Hall, F. L. and O'Neill, K. (1993) J. Immunol. **150**, 1563–1577
12 Liles, W. C., Ledbetter, J. A., Waltersdorph, A. W. and Klebanoff, S. J. (1995) J. Leukocyte Biol. **58**, 690–697
13 ElBenna, J., Faust, L. P., Johnson, J. L. and Babior, B. M. (1996) J. Biol. Chem. **271**, 6374–6378
14 Nick, J. A., Avdi, N. J., Young, S. K., Knall, C., Gerwins, P., Johnson, G. L. and Worthen, G. S. (1997) J. Clin. Invest. **99**, 975–986
15 Dziarski, R., Jin, Y. P. and Gupta, D. (1996) J. Infect. Dis. **174**, 1–7
16 Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNulty, D. F. G. T., Fisher, S., McDonnell, P. C. and Carr, S. A. (1997) J. Biol. Chem. **272**, 12116–12121
17 Cobb, M. H. and Goldsmith, E. J. (1995) J. Biol. Chem. **270**, 14843–14846
18 Kumar, S. M., M. M., McDonnell, P. C., Lee, J. C., Livi, G. P. and Young, P. R. (1995) J. Biol. Chem. **270**, 29043–29046
19 Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J. and Davis, R. J. (1995) J. Biol. Chem. **270**, 7420–7426
20 Clark-Lewis, I., Sanghera, J. S. and Pelech, S. L. (1991) J. Biol. Chem. **266**, 15180–15184
21 Brunet, A. and Pouyssegur, J. (1996) Science **272**, 1652–1655
22 Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995) Science **270**, 1326–1331
23 Read, M A., Whitley, M. Z., Gupta, S., Pierce, J. W., Best, J., Davis, R. J. and Collins, T. (1997) J. Biol. Chem. **272**, 2753–2761
24 Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A. and Pouyssegur, J. (1993) J. Cell Biol. **122**, 1079–1088
25 Wang, X. Z. and Ron, D. (1996) Science **272**, 1347–1349
26 Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R. and Landvatter, S. W. (1994) Nature (London) **372**, 739–746
27 Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7686–7689
28 Schnyder, B., Lahm, H., Woerly, G., Odartchenko, N., Ryffel, B. and Car, B. D. (1996) Biochem. J. **315**, 767–774
29 Clifford, C. B., Slauson, D. O., Neilsen, N. R., Suyemoto, M. M., Zwahlen, R. D. and Schlafer, D. H. (1989) Inflammation **13**, 221–231
30 Sengelov, H., Kjeldsen, L., Diamond, M. S., Springer, T. A. and Borregaard, N. (1993) J. Clin. Invest. **92**, 1467–1476
31 Maulik, N., Watanabe, M., Zu, Y.-L., Huang, C.-K., Cordis, G. A., Schley, J. A. and Das, D. K. (1997) FEBS Lett. **396**, 233–237
32 Zu, Y. L., Ai, Y., Gilchrist, A., Labadia, M. E., Sha'afi, R. I. and Huang, C. K. (1996) Blood **87**, 5287–5296
33 Nahas, N., Molski, T. F. P., Fernandez, G. A. and Shaafi, R. I. (1996) Biochem. J. **318**, 247–253
34 Ferrante, A., Martin, A. J., Bates, E. J., Goh, D. H., Harvey, D. P., Parsons, D., Rathjen, D. A., Russ, G. and Dayer, J. M. (1993) J. Immunol. **151**, 4821–4828
35 Jackson, S. H., Gallin, J. I. and Holland, S. M. (1995) J. Exp. Med. **182**, 751–758
36 Zhang, L. and Plow, E. F. (1996) J. Biol. Chem. **271**, 29953–29957
37 Lu, H., Smith, C. W., Perrard, J., Bullard, D., Tang, L., Shappell, S. B., Entman, M. L., Beaudet, A. L. and Ballantyne, C. M. (1997) J. Clin. Invest. **99**, 1340–1350
38 Zhou, M. J. and Brown, E. J. (1994) J. Cell Biol. **125**, 1407–1416
39 Zhang, J., Zhang, J., Shattil, S. J., Cunningham, M. C. and Rittenhouse, S. E. (1996) J. Biol. Chem. **271**, 6265–6272
40 Araki, N., Johnson, M. T. and Swanson, J. A. (1996) J. Cell Biol. **135**, 1249–1260
41 Ireton, K., Payrastre, B., Chap, H., Ogawa, W., Sakaue, H., Kasuga, M. and Cossart, P. (1996) Nature (London) **274**, 780–782
42 Baggiolini, M., Dewald, B., Schnyder, J., Ruch, W., Cooper, P. H. and Payne, T. G. (1987) Exp. Cell Res. **169**, 408–418
43 Doherty, J. B., Shah, S. K., Finke, P. E., Dorn, C. P. J., Hagmann, W. K., Hale, J. J., Kissinger, A. L., Thompson, K. R., Brause, K. and Chandler, G. O. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8727–8731
44 Thelen, M., Uguccioni, M. and Bosiger, J. (1995) Biochem. Biophys. Res. Commun. **217**, 1255–1262
45 Knall, C., Worthen, G. S. and Johnson, G. L. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 3052–3057
46 Loike, R., Kanakaraj, P. and Silverstein, B. (1995) J. Exp. Med. **184**, 1027–1035
47 Lee, J. C., Badger, A. M., Griswold, D. E., Dunnington, D., Truneh, A., Votta, D., White, J. R., Young, P. R. and Bender, P. E. (1993) Ann. N.Y. Acad. Sci. **696**, 149–170
48 Trotta, R., Kanakaraj, P. and Perussia, B. (1996) J. Exp. Med. **184**, 1027–1035

Received 27 May 1997/19 November 1997; accepted 29 December 1997