The selective enzyme inhibitors genistein and Ro 31-8220 were used to assess the importance of protein tyrosine kinase (PTK) and protein kinase C (PKC), respectively, in N-formyl-methionyl-leucyl-phenylalanine (FMLP) induced generation of superoxide anion and thromboxane B2 (TXB2) in guinea-pig alveolar macrophages (AM). Genistein (3–100 μM) dose dependently inhibited FMLP (3 nM) induced superoxide generation in non-primed AM and TXB release in non-primed or in lipopolysaccharide (LPS) (10 ng/ml) primed AM to a level > 80% but had little effect up to 100 μM on phorbol myristate acetate (PMA) (10 nM) induced superoxide release. Ro 31-8220 inhibited PMA induced superoxide generation (IC50 0.21 ± 0.10 μM) but had no effect on or potentiated (at 3 and 10 μM) FMLP responses in non-primed AM. In contrast, when present during LPS priming as well as during FMLP challenge Ro 31-8220 (10 μM) inhibited primed TXB2 release by > 80%. The results indicate that PTK activation is required for the generation of these inflammatory mediators by FMLP in AM. PKC activation appears to be required for LPS priming but not for transducing the FMLP signal; rather, PKC activation may modulate the signal by a negative feedback mechanism.

**Key words:** Alveolar macrophage activation, Cell priming, Protein kinase C inhibitors, Protein tyrosine kinase inhibitors

### Introduction

The alveolar macrophage (AM) is the most abundant cell in the airway lumen and may be an important contributor to the inflammation associated with pulmonary diseases such as asthma. Isolated AM respond to a variety of immunological and non-immunological stimuli by generating both acute-phase and pro-inflammatory mediators. The acute-phase mediators include the spasmogens thromboxane A2, leukotriene C4 and platelet activating factor (PAF) as well as reactive oxygen species and lysozymes which can cause local tissue damage. Among the pro-inflammatory mediators are PAF, leukotriene B4 and a number of cytokines which are chemotaxant to mast cells, eosinophils and lymphocytes, all of which have been implicated in pulmonary inflammation. In addition to being directly activated AM can be primed *in vitro* by agents including LPS and gamma interferon to give an exaggerated response to subsequent stimuli. AM primed *in vitro* may be comparable with AM from asthmatic subjects which have been shown to release greater amounts of many of the above mediators on stimulation than cells from non-asthmatic subjects.

The biochemical mechanisms underlying priming are poorly understood but may involve amplification of signal transduction processes. For the chemotactic peptide FMLP three receptor-coupled signalling pathways have been identified: (i) G-protein linked phospholipase C (PLC) which generates inositol 1,4,5 trisphosphate and diacylglycerol (DAG) from phosphoinositides, (ii) phospholipase D generation of DAG from phosphatidyl choline, and (iii) activation of non-receptor PTK. As PTK can activate PLC to generate DAG, activation of PKC could represent a common end point for all of these pathways, although other mechanisms of PTK-dependent cell activation clearly exist. Both PKC and PTK have been implicated in the priming process in macrophages; for instance in murine peritoneal macrophages LPS priming stimulated myristoylation and translocation of an intracellular substrate for PKC thus improving the efficiency of this signal transduction pathway, whilst LPS priming in P388 D1 cells was shown to require protein synthesis and to be blocked by a PTK inhibitor.

In order to investigate the importance of PTK and PKC enzyme systems in FMLP activation of AM we have studied the effects of the selective PTK inhibitor genistein and the selective PKC inhibitor Ro 31-8220 on superoxide and TXB2 (as a marker for arachidonate metabolism) generation in normal cells, and on TXB2 generation in LPS primed cells. As a control measure the effects of these compounds on superoxide release induced by FMLP were compared with those on superoxide release induced...
by the phorbol ester PMA, which is known to act by stimulating PKC directly.

Materials and Methods

Male Dunkin–Hartley guinea-pigs were purchased from David Hall, Burton-on-Trent, UK. Tissue culture reagents were obtained from Gibco; FMLP, phorbol myristate acetate (PMA), type III cytochrome c, LPS (E. coli 055:B5) and superoxide dismutase from Sigma, UK; and TXB2 radioimmunoassay kits from Amersham, UK. Protein was determined after disruption of cells with 0.1% Triton X-100 using BioRad Coomassie blue reagent. Genistein was purchased from ICN-Flow whilst Ro 31-8220 was synthesized at blue reagent. Genistein was purchased from Amersham, UK.

Preparation of AM: Male guinea-pigs (400–600 g) were killed by intraperitoneal administration of sodium pentobarbitone, the trachea cannulated and bronchoalveolar lavage carried out with two 5 ml volumes of saline at 37°C. Lavage fluid was centrifuged at 300 × g for 8 min, cells resuspended in Ca2+ and Mg2+ free HBSS and pooled. The cell suspension was centrifuged again and the cell resuspended at 106 cells/ml in RPMI 1640 medium supplemented with 1% foetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. For superoxide experiments cells were plated out in 24-well dishes at 106 cells/well; for TXB2 experiments in 96-well dishes at 2 × 105 cells/well. After 2 h in an incubator (37°C, 5% CO2) non-adherent cells and debris were removed by changing the medium. Adherent AM were left in the incubator for 18–20 h before use.

Measurement of superoxide production: Tissue culture medium was removed, AM washed once with HBSS then HBSS (0.5 ml) with or without inhibitors was added and the cells incubated at 37°C for 2 h. Buffer was then replaced by fresh HBSS (0.5 ml) containing Fe3+ cytochrome c (55 μM), again with or without inhibitors. After 15 min at 37°C FMLP or PMA was added and the incubation continued for a further 30 min. Superoxide generation was determined from the difference in absorbance of the AM supernatants at 550 nm in comparison with supernatants from cells stimulated in the presence of superoxide dismutase (0.1 mg/ml). Using this protocol the absorbance difference was directly proportional to superoxide concentration.

Measurement of TXB2 release: After removal of tissue culture medium AM were washed once with HBSS then incubated for 2 h at 37°C in HBSS (0.2 ml) with or without inhibitors and with or without LPS (10 ng/ml) for cell priming. The buffer was replaced by fresh HBSS (0.2 ml) again with or without inhibitors, and the incubation continued for 15 min prior to adding FMLP for a further 15 min. Supernatants removed at appropriate times were stored at −20°C prior to quantification of TXB2 by radioimmunoassay. Samples were diluted to fall within the assay standard curve; concentrations were calculated by interpolation using a Multi Calc software program (Wallac, Finland).

Results

Effects on superoxide generation: Adherent guinea-pig AM prepared as described showed a very low basal generation of superoxide (≤0.1 nmole/assay). Both FMLP and PMA (0.3–30 nM) caused concentration dependent stimulation of superoxide generation with EC50 values of 1.2, 2.6 nM and 3.0, 5.4 nM, respectively, in two experiments and similar maximal stimulation (1–2 nmoles/assay). The effects of genistein and Ro 31-8220 were evaluated against sub-maximal (approx. EC20) agonist concentrations (3 nM FMLP, 10 nM PMA) that gave similar responses (compare Figs 1A and 1B). Consistent with its activity as a PKC antagonist Ro 31-8220 inhibited PMA induced superoxide generation (Fig. 1A); in three experiments the IC50 was 0.21 ± 0.10 μM. Whereas genistein at concentrations up to 100 μM had little effect on the PMA response it dose-dependently inhibited FMLP induced superoxide generation (Fig. 1B); in four experiments the IC50 was 54 ± 9 μM. Ro 31-8220 at low concentrations (0.1–1 μM) had little or no inhibitory effect on the FMLP response whereas at higher concentrations (3 and 10 μM) it consistently enhanced the FMLP response (by 64 ± 16% at 10 μM; n = 3 experiments).

At the highest concentrations employed Ro 31-8220 and genistein did not reduce cell viability as assessed by trypan blue dye exclusion, viability being consistently ≥95%.

Effects on TXB2 release: Pre-incubation of guinea-pig AM for 2 h with LPS (10 ng/ml) in itself caused some increase in TXB2 release from a basal level of
Inhibition of alveolar macrophage activation

1.50
1.25
1.00
0.75
0.50
0.25
0.00
\( \text{(A)} \)

FIG. 1. The effects of Ro 31-8220 and genistein on PMA (Panel A) or FMLP (Panel B) induced superoxide release from non-primed guinea-pig AM. AM (10^5/well) were incubated with or without inhibitors for 2.25 h at 37°C prior to addition of stimulus for 30 min. Superoxide was determined by superoxide dismutase inhibitable reduction of Fe^{3+} cytochrome c. Mean ± S.E.M. (n=3) from a single representative experiment.

0.10 ± 0.01 to 1.0 ± 0.2 ng/10 μg AM protein (n = 4 experiments). However LPS potentiated markedly (primed) the response to FMLP over the full concentration range tested (0.3–30 nM) (Fig. 2). FMLP (3 nM), as a non-maximal stimulus, was used to study the effects of Ro 31-8220 and genistein on TXB\(_2\) release.

When present during the 2 h pre-incubation, or 2 h LPS priming, stage and the subsequent challenge stage genistein (1–30 μM) dose-dependently inhibited FMLP stimulated TXB\(_2\) release in both non-primed and primed AM with similar IC\(_{50}\) values (17 and 18 μM, respectively, in the experiment shown in Fig. 3A). Under these conditions Ro 31-8220 (0.1–10 μM) did not inhibit FMLP induced TXB\(_2\) release in non-primed AM but tended rather to potentiate release at higher concentrations (by >27% at 3 and 10 μM in the experiment shown) (Fig. 3B). The extent of potentiation varied between experiments but was a consistent observation. In LPS primed AM low concentrations of Ro 31-8220 (0.1–3 μM) had no effect on the FMLP response whereas Ro 31-8220 at 10 μM inhibited TXB\(_2\) release by 80% relative to the LPS baseline level. Since this suggested that Ro 31-8220 had an inhibitory effect on a component of LPS priming, further studies were performed in which Ro 31-8220 (10 μM) was added to the AM only during selected stages of priming and/or activation (Fig. 4). Ro 31-8220 had no effect on the LPS primed response when present only during FMLP challenge, inhibited the response by approx. 50% when present only during priming, but had greatest effect, reducing FMLP induced TXB\(_2\) release by 80 and 100% in relation to the LPS baseline value (n = 2 experiments), when present during both priming and challenge. The essential requirement for inhibition was thus that Ro 31-8220 was present during the LPS priming stage.

Discussion

Ro 31-8220 and genistein were used in the present study as selective inhibitors of PKC and PTKs, respectively. Ro 31-8220 is reported to have a 100-fold selectivity for PKC over protein kinase A (PKA) and a 1,000-fold selectivity over Ca\(^{2+}\)-calmodulin dependent kinase (CAMK) and has been used by several groups as a selective PKC inhibitor in intact cells. Genistein inhibits a number of PTKs but...
FIG. 3. The effects of genistein (Panel A) or Ro 31-8220 (Panel B) on FMLP induced TXB2 release from non-primed or LPS-primed AM. AM were primed with LPS (10 ng/ml) and stimulated with FMLP (3 nM) as described in the legend to Fig. 2. Genistein or Ro 31-8220 were added during both LPS priming and FMLP stimulation. Mean ± S.E.M. (n = 4) from a single representative experiment. □, Non-primed; ■, primed.

FIG. 4. Influence of the stage of LPS priming, or FMLP challenge at which Ro 31-8220 was added, on its ability to inhibit TXB2 release. AM were primed with LPS (10 ng/ml) and stimulated with FMLP (3 nM) as described in the legend to Fig. 2. LPS + (Ro + FMLP): Ro 31-8220 added for 15 min prior to, and during FMLP stimulation, but not during priming; (Ro + LPS) + FMLP: Ro 31-8220 added during LPS priming but removed before FMLP stimulation; (Ro + LPS) + (Ro + FMLP): Ro 31-8220 added during LPS priming and for 15 min prior to, and during, FMLP stimulation. Mean ± S.E.M. (n = 4) from a single representative experiment.
that LPS can activate PKC in macrophages\textsuperscript{26} and that responses to LPS such as cytokine production are blocked by PKC inhibitors.\textsuperscript{25} It does, however, differ from findings in P388 D1 macrophages where the non-selective PKC inhibitor H7 did not affect cell priming.\textsuperscript{16} The fact that Ro 31-8220 present during FMLP challenge as well as during priming increased the inhibition of TXB\textsubscript{2} release contrasts with the potentiation of release seen in non-primed AM and probably reflects the complex interplay between feed forward and feed back effects of PKC on mediator release in these cells.

In summary, using selective enzyme inhibitors it was found that activation of PTK is a key event in FMLP generation of superoxide and TXB\textsubscript{2} in guinea-pig AM whereas PKC either is not activated, or is activated but has counter-balancing feed back and feed forward effects on mediator release in non-primed cells. On the other hand activation of PKC appears to be required for LPS priming of these cells for enhanced TXB\textsubscript{2} release. Thus both PTK and PKC inhibitors could conceivably be effective in reducing the release of these mediators from macrophages in the airways in vivo under conditions where priming has occurred during an inflammatory response.

References


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