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INHIBITION BY FK506 OF FORMYL PEPTIDE-INDUCED NEUTROPHIL ACTIVATION AND ASSOCIATED PROTEIN SYNTHESIS

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Abstract—The macrolide FK506 inhibited, by up to 50%, neutrophil migration and the production of the superoxide radical in response to the formyl peptide, formyl-methionyl-leucyl-phenylalanine (FMLP). The production of the superoxide radical in response to phorbol 12-myristate 13-acetate (PMA) was unaffected by FK506. The inhibition of neutrophil functions was accompanied by a partial reversal of FMLP-induced synthesis of cellular proteins, despite a rise in intracellular Ca^{2+}. Neutrophils treated with FK506 demonstrated a small (average 23%) though significant decrease in formyl-peptide receptor numbers but receptor binding affinity was unaffected. The effects of FK506 on neutrophil activation appear to be analogous to those in T-lymphocytes. The incomplete inhibition, by FK506, of neutrophil responses suggests further that activation by FMLP is mediated via distinct multiple signalling pathways, including protein kinase activation and protein synthesis. The inability of FK506 to reduce FMLP-induced rises in cellular Ca^{2+} or PMA-induced activation of neutrophils suggests that its action is distal to Ca^{2+} mobilization and distinct from pathways relying on PKC activation. Thus the immunosuppressive effects of FK506 *in vivo* might be mediated through the inhibition of inflammatory cells other than lymphocytes and the drug therefore has therapeutic potential in a variety of inflammatory conditions. The drug also has potential *in vitro* for the characterization of signalling pathways from the plasma membrane to the nucleus.

Key words: formyl-peptide; receptor; immunophilin; signalling

The macrolide antibiotic FK506 is a potent immunosuppressive agent with potential for the treatment of allograft rejection and other inflammatory conditions [1–4]. The mechanism of action of FK506 has received considerable attention, particularly concerning its effects on T-lymphocytes. The action of FK506 is effected by complexes between the drug and its intracellular binding protein, FKBP [5]. The FK506–FKBP complex is thought to suppress T-lymphocyte activation at an early stage (G_0–G_1), resulting in the inhibition of transcription of lymphokines including interleukin-2, interleukin-3, interleukin-4, granulocyte-macrophage colony-stimulating factor, tumour necrosis factor and interferon-γ [6, 7]. Thus FK506 intercepts intracellular signals from the T-cell antigen receptor to the nucleus. The drug has been shown to inhibit induced interleukin-2 production by, and proliferation of, T-cells without affecting rises in intracellular $[Ca^+]$, indicating that the action of FK506-FKBP occurs after initial membrane stages of cell signalling involving generation of second messengers [8]. More recently, the inhibition, by FK506, of T-lymphocyte activation has been placed at serine/threonine dephosphorylation events critical for passage through G_1 [9]. Furthermore, it has been demonstrated that FK506–FKBP complexes inhibit the translocation of a transcription factor (NF-AT) from the cytosol to the nucleus [10], and the target molecule of these complexes appears to be the phosphatase calcineurin [11–13]. This action would agree well with the data showing that inhibition of lymphocyte activation by FK506 is at an early stage, i.e. G_0–G_1, but follows initial transmembrane signalling events.

Despite the apparently ubiquitous expression of the FKBP [14, 15], the action of FK506 is still often considered to be specific to T-cells [16]. The immunosuppressive activity of FK506 may not, however, be restricted to its effects on lymphocytes and its clinical potential might therefore currently be understated. The drug has also been shown to inhibit interleukin-1α production by macrophages [17] and to effectively block histamine release from basophils [18]. Furthermore, FK506 has been shown to reduce neutrophil infiltration in an animal model [19] and can reduce the migration of neutrophils on vitronectin substrate in response to FMLP** [20].

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** Abbreviations: FMLP, formyl-methionyl-leucyl-phenylalanine; F-Nleu-Leu-Phe-Nleu-Tyr-Lys, *N*-formyl-norleucyl - leucyl - phenylalanyl - norleucyl - tyrosyl - lysine; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

The potential clinical significance of these data justifies further investigations of the effects of this drug on neutrophil functions. Neutrophils have been implicated in the pathological changes associated with a variety of diseases including liver allograft rejection [21, 22]. Agents that can inhibit neutrophil activation and recruitment to tissues therefore have considerable clinical potential.

In this paper we demonstrate that FK506 can inhibit directly the activation of human neutrophil functions in response to FMLP, which has been shown to stimulate neutrophils via activation of phospholipase C, with the production of IP_3 and diacylglycerol [23]. Thus both calcium- and PKC-dependent pathways are invoked by FMLP. FK506 did not reduce the rise in intracellular calcium and did not inhibit activation by the phorbol ester PMA. Our data therefore suggest that only Ca^{2+}-dependent, PKC-independent processes are affected by FK506. The effect of FK506 on neutrophil activation was associated with a partial reversal of FMLP-induced protein synthesis, suggesting that the mechanism of action may be analogous to that in T-cells.

MATERIALS AND METHODS

Isolation of blood neutrophils. Venous blood was obtained from healthy volunteers and the neutrophils isolated by one of two procedures, both using centrifugation on isotonic discontinuous Percoll gradients [24]. All solutions were prepared in pyrogen-free injectable water (Phoenix Pharmaceuticals, Gloucester, U.K.) and reagents were confirmed to contain <20 ng/L endotoxin using the Kabi Vitrum Coatest (ICN-Flow Laboratories, Rickmansworth, U.K.). For cell migration experiments, each blood sample was anticoagulated with lithium heparin, diluted with an equal volume of 0.15 M NaCl solution and 5 mL layered on to two solutions of Percoll (Pharmacia Biosystems Ltd, Milton Keynes, U.K.) consisting of 3 mL 1.096 g/mL Percoll beneath 2 mL of 1.075 g/mL Percoll. The tubes were centrifuged at 400 *g* for 20 min at room temperature and the neutrophils harvested from the interface of the two Percoll layers. The cells were washed twice in Tris-buffered RPMI 1640 medium (ICN-Flow Laboratories), counted and resuspended in medium. The leucocytes present were 96% neutrophils, which were 98% viable as assessed by exclusion of trypan blue. This procedure resulted in the presence of some erythrocytes which, although irrelevant in migration assays, were considered unacceptable for the other experiments, particularly the analysis of cellular proteins. For these, therefore, the venous blood (20 mL) was collected into tubes containing 26 mg ethylenediamine tetraacetic acid (BDH, Poole, U.K.). To each tube of anticoagulated blood, 2 mL of 6% (w/v) Dextran (average M_r 485,000) solution was added. The solutions were mixed gently and the erythrocytes allowed to sediment under gravity for 1 hr at room temperature. The leucocyte-rich suspension was collected, centrifuged, and washed in 0.15 M NaCl solution, and the neutrophils isolated on Percoll gradients as described above. Preparations containing erythrocytes were discarded. Dextran sedimentation was not used to prepare neutrophils for migration experiments because we have found that this procedure can inhibit neutrophil mobility.

Formyl-peptide induced neutrophil migration. The migratory response of neutrophils was assessed using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, CA, U.S.A.), as described by Falk *et al.* [25]. Neutrophils isolated by Percoll gradients were suspended at 1.5×10^6 cells/mL in HEPES-buffered RPMI-1640 medium with 2% (w/v) BSA. Samples of these cell suspensions (50 μL) were placed in the upper chambers of the microchemotaxis apparatus, which were separated from the lower chambers by a 2 μm pore-size polycarbonate membrane (Nucleopore, Pleasanton, CA, U.S.A.). The lower chambers contained FMLP (Sigma Chemical Co., Poole, U.K.), 10 nM, in medium. Samples of neutrophil preparations from six subjects were pre-incubated for 2 hr with FK506 (supplied by Fujisawa, Munich, Germany) at concentrations of 0.005–50.0 ng/mL, diluted from a stock solution in methanol, into HEPES-buffered RPMI 1640 with 2% (w/v) bovine serum albumin. Control cells were preincubated with medium only. Each sample was assayed in triplicate and each assay included negative controls in which FMLP was excluded from the lower chambers. The chambers were incubated for 90 min at 37°, after which the non-migrating cells were removed from the upper surface of the polycarbonate membrane with a rubber scraper. The membranes were fixed, stained with Diff-Quik (Travenol, Newbury, U.K.) and the number of cells on the lower surface was counted (five microscope fields at ×400 magnification). The average number of cells/field was calculated and the mean value of triplicate assays determined.

Superoxide anion (O_2^-) *production.* The neutrophils from five subjects were incubated in Linbro culture plate wells (10^6 cells/well) in medium alone or with FK506 for 1 hr, 37°, at concentrations ranging from 0.005 to 50.0 ng/mL. The assay medium was 1 mL of 0.15 mol/L phosphate-buffered saline with 1.24 mg cytochrome *c* (Sigma Chemical Co.) and 1 mM each of $MgCl_2$ and $CaCl_2$. Each cell preparation was incubated for 2 hr at 37° with either 1 μM FMLP or 16 nM phorbol 12-myristate 13-acetate (PMA). After incubation, the contents of each well were centrifuged and reduction of cytochrome *c* measured by spectrophotometry at 550 nm. Excess superoxide dismutase (Sigma Chemical Co.) was added to duplicates of samples in order to catalyse the decomposition of O_2^- to H_2O_2 and provided a blank for subtracting the reduction of cytochrome *c* due to agents other than O_2^-. Additional blanks were represented by wells containing no cells.

Intracellular Ca^{2+}. The effect of FK506 on FMLP-induced neutrophil intracellular Ca^{2+} concentrations was investigated using the method of Bijsterbosch *et al.* [26]. Isolated neutrophils, preincubated for 30 min with or without 1 ng/mL FK506, were suspended at 10^7 cells/mL in RPMI 1640 medium with 10% (w/v) foetal calf serum and 1 μM of the calcium-dependent fluorescent dye Fura 2-AM (Gibco-BRL Ltd, Uxbridge, U.K.). The cells were incubated at 37° in 5% CO_2/95% air for 60 min,

washed in Hanks' buffered saline with 10% foetal calf serum and 10 mM HEPES (pH 7.2), resuspended at 2×10^6 cells/mL and stored on ice. The cells were warmed to 37° for 5–10 min before measurement of intracellular Ca^{2+}, in response to the addition of 1 μM FMLP, using a Hitachi Model F-2000 intracellular cation measurement system.

Formyl-peptide receptors. Formyl-peptide receptor analysis was by a modification of the method described by Niedel *et al.* [27]. The peptide F-Nleu-Leu-Phe-Nleu-Tyr-Lys (Sigma Chemical Co.) was iodinated by the chloramine-T method [28] using [^{125}I]iodide (ICN-Flow, High Wycombe, U.K.). BSA (Sigma Chemical Co.), 1% (w/v), was added to the ^{125}I-F-Nleu-Leu-Phe-Nleu-Tyr-Lys and the solution stored at −70°. Neutrophils (2×10^5/tube), isolated from six healthy volunteers as described above, were incubated for 30 min at 37° with or without 1 ng/mL FK506, centrifuged and incubated for a further 30 min at 4° in 0.1 mL of RPMI 1640 medium with 0.1 g/mL BSA, 5 μg/mL cytochalasin B and 0.65 mg/mL sodium azide (all reagents from Sigma Chemical Co.). These cells were dispensed into microfuge tubes and incubated (in duplicate) with ^{125}I-F-Nleu-Leu-Phe-Nleu-Tyr-Lys at concentrations ranging from 7.5 to 240 nM. Control preparations also contained unlabelled formyl peptide at 2000× the concentrations of ^{125}I-F-Nleu-Leu-Phe-Nleu-Tyr-Lys in order to deduct counts due to "non-specific" binding. The cell suspensions were incubated for 1 hr at 4° and then 0.1 mL phthalate oil (1.5 vol. di-*n*-butyl phthalate: 1 vol. di-iso-octyl phthalate, from BDH Chemicals, Dorset, U.K.) was added to each of the tubes, which were centrifuged for 1 min in a microfuge and frozen to −20°. Each frozen tube was cut at the oil layer and the cell pellets and supernatants were assayed separately for ^{125}I using an LKB Multigamma counter. The number of counts bound to control cells in the presence of excess formyl peptide (non-specific binding) was deducted from test results and the number of receptors/cell and dissociation constant (K_d) were calculated by Scatchard analysis [29]. Differences between FK506-treated and control cells were tested by Student's *t*-test for paired data.

Analysis of cellular proteins. Neutrophils were metabolically labelled with [^{35}S]methionine: cysteine (75%:15%; TRANS ^{35}S-label, ICN Biochemicals) at 50 μCi/mL for 4 hr in methionine-free RPMI 1640 medium (ICN Biochemicals). The neutrophils were then suspended at 2×10^6 cells/mL in RPMI 1640 medium and incubated with or without 1 ng/mL FK506 for 1 hr, before the addition of FMLP (1 μM) for 2 hr. Control incubations in the absence of FMLP and with FK506 alone were also performed. Incubations were terminated by pelleting the cells (8000 *g*; 1 min) and extracting the proteins directly into 60 μL of isoelectric focusing (IEF) sample buffer containing 9 M urea, 2% v/v 3-[(3-cholamidopropyl) dimethylammoniol]-1-propanesulphonate (Sigma Chemical Co.), 80 mM dithiothreitol (Sigma Chemical Co.). Ampholines (5% v/v, pH 3.5–10) (Millipore U.K. Ltd) and 4% v/v Nonidet P-40 (Sigma Chemical Co.). Proteins were extracted at room temperature for at least 1 hr and insoluble material was removed by centrifugation (8000 *g*, 1 min) prior to two-dimensional electrophoresis.

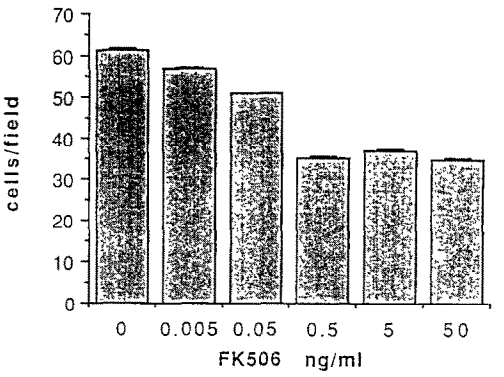

Fig. 1. The effects of increasing concentrations of FK506 on the migratory response of human neutrophils to 10 nM FMLP. Results are shown as average number of cells/microscope field (+SEM) on the chemotaxis membranes (N = 6).

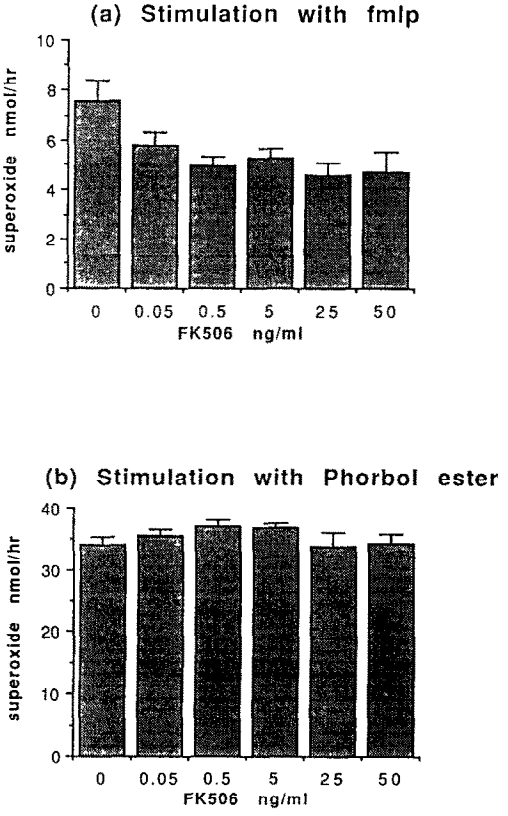

Fig. 2. Production of superoxide anion by neutrophils, preincubated with FK506, in response to (a) 1.0 μM FMLP, and (b) 16 nM PMA. Results are shown as average nmol O_2^-/hr (+SEM) by cells from five subjects.

Two-dimensional gel electrophoresis. ^{35}S-Labelled cellular proteins were separated by two-dimensional gel electrophoresis essentially as described by O'Farrell [30], using a Millipore Investigator 2-D

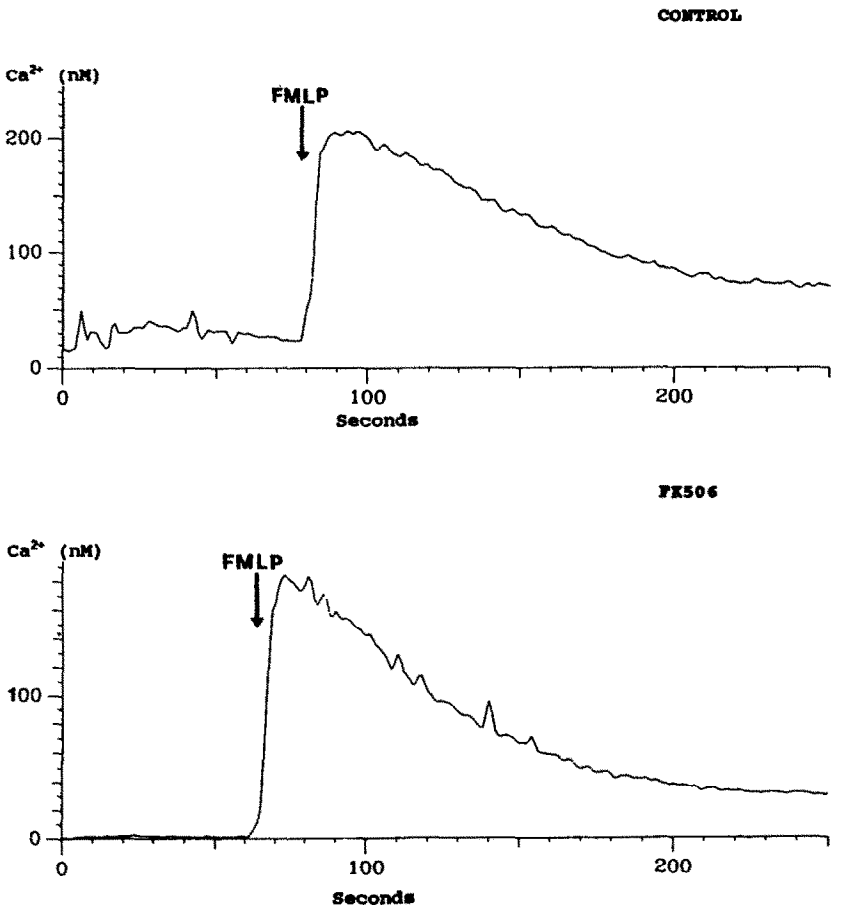

Fig. 3. Neutrophil intracellular Ca^{2+} concentrations in response to 1.0 μM FMLP in the absence of FK506 (upper trace) or after preincubation with 1 ng/mL FK506 (lower trace).

gel electrophoresis system. Samples were assayed for protein using a commercial kit (Biorad) modified for samples containing urea and ampholines [31] and gels were loaded with equivalent amounts of protein (100 μg). Isoelectric focusing was performed for 18,000 Vhr in gels containing 3% (v/v) Ampholines (pH 3.5–10, Millipore). The second dimension consisted of SDS–PAGE (12% acrylamide gel) according to Laemmli (1970). Gels were prepared for fluorography using Amplify (Amersham International plc) and ^{35}S-labelled proteins were detected in dried gels by autoradiography at −70° using pre-flashed film (HyperFilm-βmax, Amersham International plc).

RESULTS

Formyl-peptide induced neutrophil migration

The migratory response of human neutrophils to 10 nM FMLP was significantly reduced ($P < 0.001$; ANOVAR) by preincubation of the cells with increasing concentrations of FK506, from the control (no FK506) value of 61 cells/field (SEM 0.32). Maximum inhibition (average 42%) was achieved with 0.5 ng/mL FK506 to, on average, 35 cells/field (SEM 0.27). Inhibition was maintained, but not increased further, with FK506 concentrations in excess of 5 ng/mL (Fig. 1). In the absence of FMLP the average number of cells migrating was 13.2/field (SEM 1.7).

Superoxide anion (O_2^-) production

Stimulation of neutrophils with 1 μM FMLP increased significantly the production of superoxide anion from a "resting" value of 1.68 nmol/10^6 cells/hr (SEM 0.29) to 7.6 nmol/10^6 cells/hr (SEM 0.8). The production of superoxide anion by neutrophils in response to FMLP was significantly inhibited by FK506 ($P < 0.001$; ANOVA). As with cell migration, maximum inhibition (average 35%; mean 5.0 nmol/10^6 cells/hr, SEM 0.4) was achieved at an FK506 concentration of 0.5 ng/mL, with inhibition sustained but not increased at higher concentrations (Fig. 2a). Incubation of the neutrophils with PMA resulted in greater amounts of superoxide anion production (mean 34.0; SEM 1.4 nmol/10^6 cells/hr) than with FMLP but preincubation of the cells with FK506 had no effect (Fig. 2b).

Intracellular Ca^{2+}

The addition of 1 μM FMLP to neutrophils resulted in a rapid rise in intracellular Ca^{2+} (Fig. 3). Preincubation of neutrophils with 1 ng/mL FK506 had no effect on the rate or magnitude of the rise in intracellular concentration of Ca^{2+} (Fig. 3).

Formyl-peptide receptors

Scatchard analysis of ^{125}I-F-Nleu-Leu-Phe-Nleu-Tyr-Lys binding to neutrophils (Fig. 4a) suggested the presence of a single class of receptor (mean 265,000 receptors/cell; SEM 41,300) with average K_d of 4.2 pM (SEM 0.89 pM). After 30 min incubation with 1 ng/mL FK506 (Fig. 4b), the receptor numbers were, on average, 204,000/cell (SEM 24,500; P = 0.045) and the average K_d was 4.8 pM (SEM 0.29 pM; P = ns).

Protein synthesis

Two-dimensional electrophoresis gels of ^{35}S-labelled cellular proteins revealed the increased expression, compared to control cells (Fig. 5A), of several proteins in response to 2 hr incubation with FMLP; these are labelled 1–7 on Fig. 5B: 1 = M_r 40,000; 2 = 80,000; 3 = 43,000–46,000; 4 = 41,000; 5 = 40,000; 6 = 23,000–27,000; 7 = 28,000. Two proteins, labelled 4 and 5 in Fig. 5B, were not detected in control cells but appeared to be expressed *de novo* upon stimulation with FMLP. Pre-incubation of the neutrophils with FK506 reversed the majority of changes in protein expression associated with FMLP. The exceptions were proteins 1 and 7 (Fig. 5C). Incubation of the cells with FK506 alone (data not shown) caused an increase in the expression of four proteins; two of M_r 40,000, one of 41,000 and one of 43,000. These proteins are labelled with asterisks in Fig. 5C. The results are representative of three experiments.

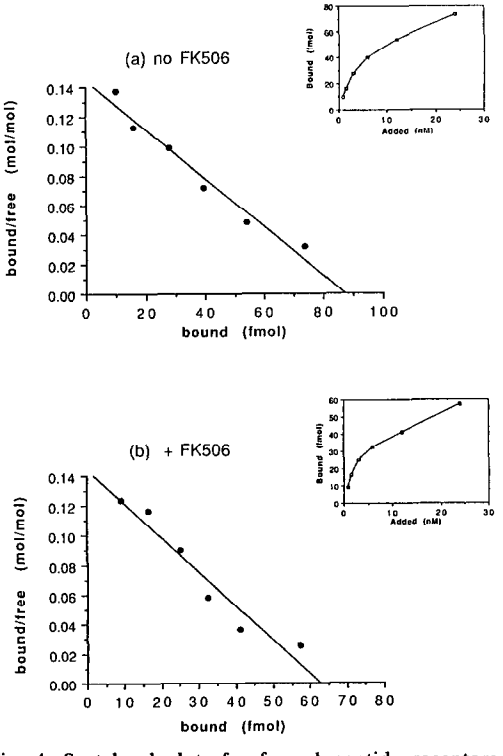

Fig. 4. Scatchard plots for formyl peptide receptors on neutrophils; (a) in the absence of FK506, and (b) after preincubation with 1 ng/mL FK506. Each point represents the average results from six preparations of cells. The inset graphs show the binding curves for ^{125}I-F-Nleu-Leu-Phe-NLeu-Tyr-Lys over the corresponding range of radiolabelled ligand from which the Scatchard plots were derived.

DISCUSSION

The immunosuppressive properties of FK506 and its efficacy as an inhibitor of allograft rejection have been ascribed largely to the inhibition of T-lymphocytes. Effector cells other than lymphocytes have, however, been implicated in the process of rejection [22, 32, 33]. The ability of FK506 to inhibit macrophage, basophil and neutrophil functions [17, 18, 20] suggests that the therapeutic effects of the drug might also result partly from its effects on non-lymphocytic effector cells. These observations indicate a wider potential therapeutic role for FK506.

In the present paper we have investigated the effects of FK506 on FMLP-induced neutrophil activation. Partial but significant inhibition of superoxide production and chemotactic responses were observed with concentrations of FK506 which were within the ranges achieved in the blood of patients administered the drug *in vivo* [34]. Hendey *et al.* [20] reported that FK506 at 0.1 μg/mL inhibited, by about 80%, FMLP-stimulated neutrophil migration on substrates coated with vitronectin, but not those coated with fibronectin or albumin. The conclusion was that FK506 inhibited specifically integrin-mediated adhesion to vitronectin. Our results demonstrated FK506-mediated inhibition of cell migration and superoxide radical production in the presence of albumin, but with only 50% maximum inhibition at FK506 concentrations between 0.05 and 0.5 ng/mL. Our results do not, therefore, support such a specific requirement for vitronectin. These differences may result from the use of distinct experimental methods. Hendey *et al.* [20] employed a different type of chemotaxis chamber and high concentrations of FK506. Furthermore, they isolated neutrophils on Ficoll-hypaque gradients, which have been shown to activate these cells and potentially affect functional assays [35]. Further work may clarify the role of integrin modulation in the action of FK506 on neutrophils.

It is possible that some inhibitors of FMLP-induced neutrophil responses may interfere with FMLP-receptor interactions, as described with the tyrosine kinase inhibitor ST 638 [36]. We therefore measured formyl-peptide receptor affinity and numbers in cells treated with FK506. The numbers of formyl-peptide receptors were of the same order as those reported by others using the ligand ^{125}I-F-Nleu-Leu-Phe-Nleu-Tyr-Lys, which generally gives higher numbers of receptors per cell than tritiated

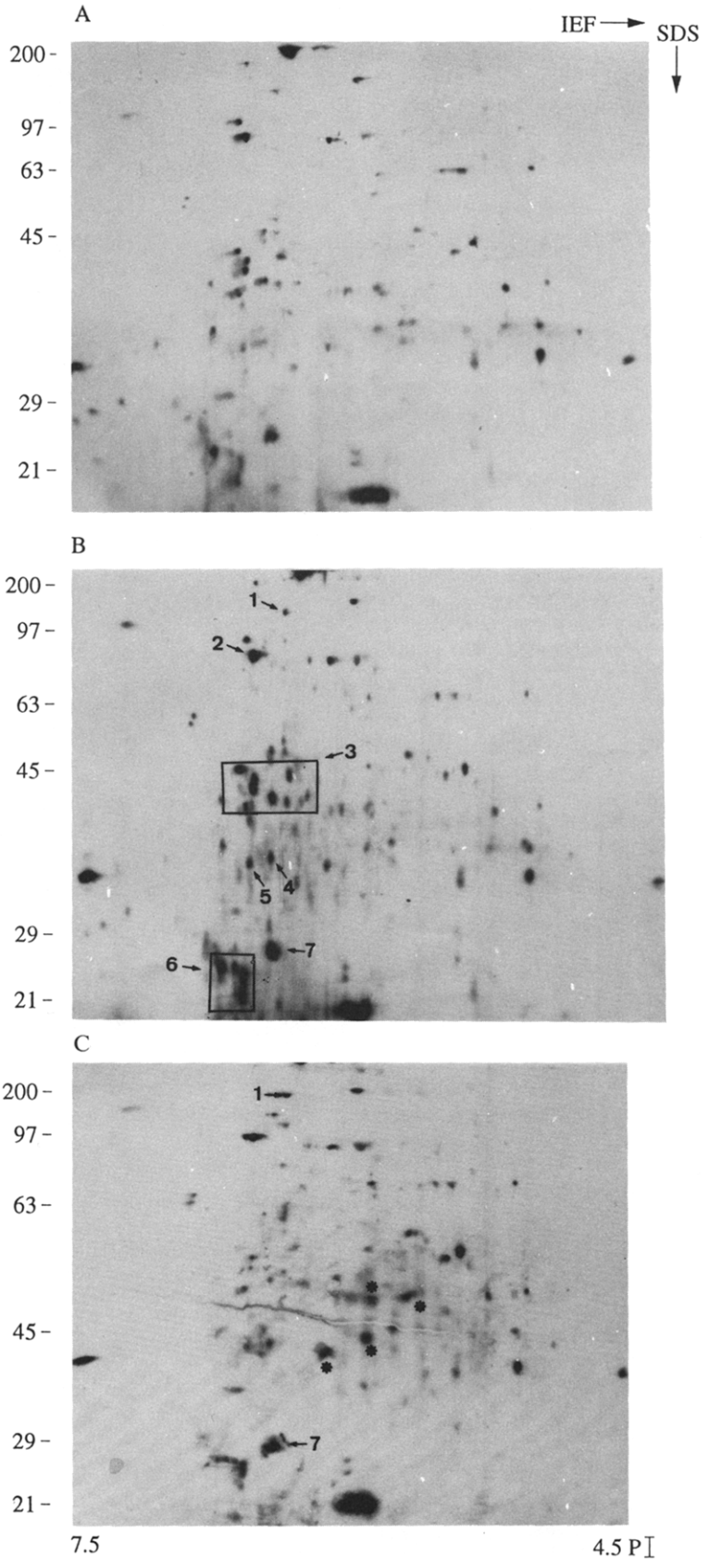

Fig. 5. Autoradiographs of two-dimensional electrophoresis gels of cellular proteins from neutrophils labelled with [^{35}S]methionine: (A) control (untreated) neutrophils; (B) neutrophils incubated with 1 μM FMLP; (C) neutrophils incubated with 1 μM FMLP and 1 ng/mL FK506.

ligand [27, 37]. The small but statistically significant reduction in formyl-peptide receptor numbers in cells incubated with FK506 might indicate that the drug alters the cell response to FMLP as a result of receptor modulation, leading to alternative pathways of intracellular signalling. Nevertheless, the change in receptor numbers was modest and receptor affinity was unaffected. We therefore favour the explanation that the effect on receptor numbers was a secondary consequence of late events in cell signalling due to FK506.

Our observations suggest that FK506 can inhibit FMLP-induced, but not PMA-induced, activation of neutrophil functions, although the site of action of FK506 is after the initial rise in intracellular Ca^{2+}. It is known that FMLP increases intracellular Ca^{2+} and activates PKC as a result of activation of phospholipase C [23]. Merritt *et al.* [38] showed that some isoenzymes of PKC, specifically those isoenzymes which are Ca^{2+} dependent, are involved in neutrophil activation. The lack of effect of FK506 on PMA activation of neutrophils indicates that the PKC signalling pathway cannot be modulated by FK506. Thus the rise in intracellular Ca^{2+} is able to activate the Ca^{2+}-dependent PKC isoenzymes and will also initiate a distinct signalling pathway. This second pathway mediates the *de novo* synthesis of specific proteins and can be inhibited by FK506. The elements of this signalling pathway need to be established in neutrophils, but may be analagous to those in T-cells.

It has been shown that FK506 inhibits T-cell activation as a result of the FK506-FKBP complex interacting with the protein phosphatase, calcineurin [11, 12]. This results in failure of translocation of the transcription factor NF-ATc to the nucleus and consequent failure to assemble the transcription factor complex, NF-ATc/NF-ATn [10, 39], thus blocking the transcription of proteins, including cytokines, that are normally synthesized as a requirement or consequence of T-cell activation [6, 7]. If FK506 is indeed working in a similar way in neutrophils, an effect on protein transcription could be predicted. We confirm recent observations of increased synthesis of several proteins accompanying activation of neutrophils by FMLP [40], and have shown that the inhibition of cell activation by FK506 was accompanied by a partial reversal of the associated specific changes in protein synthesis. Neutrophils have traditionally been considered as cells with little potential for protein synthesis. These data, however, confirm that mature blood neutrophils retain the ability to synthesize specific proteins in response to activating factors. The ability of FK506 to ablate partially this cellular response may underlie its inhibition of neutrophil activation. The fact that protein synthesis, in response to FMLP, was not completely ablated may explain the partial inhibition of neutrophil activation by FK506. Presumably only those genes regulated by calcineurin would be affected. The significance of the increased expression of four proteins by neutrophils treated with FK506 alone is not known. Since rapamycin also binds to FKBP [5] it is possible that this drug will also affect neutrophils in a manner similar to FK506, but this has yet to be determined.

In conclusion, the effects of FK506 on neutrophil activation suggest that the mechanism of action may be analogous to that in T-cells. It has yet to be confirmed, however, whether the inhibition of protein synthesis associated with FMLP activation is, as in lymphocytes, at the transcriptional level. Neutrophil migration in response to FMLP has been shown to be inhibited by a specific peptide inhibitor of calcineurin [20], and this phosphatase is therefore likely to be the target molecule for FK506-mediated inhibition of neutrophil activation. It also remains to be determined whether FK506 can block the activation of neutrophils by other factors, such as cytokines. Beaulieu *et al.* [40] reported that only three of 14 known neutrophil agonists tested; FMLP, tumour necrosis factor-α and granulocyte-macrophage colony-stimulating factor, induced *de novo* RNA and protein synthesis in these cells. If FK506 acts only by blocking DNA transcription and cannot prevent neutrophil responses elicited by modulation of protein phosphorylation, it is possible that this drug might also inhibit neutrophil activation by tumour necrosis factor and granulocyte-macrophage colony-stimulating factor. Conversely, FK506 would not be expected to inhibit neutrophil activation by those agonists, including several interleukins, leukotriene B4 and C5a, which do not affect protein synthesis.

The partial inhibition of neutrophil activation by FK506 and its apparent inability to affect PMA activation confirm the suggestion [41, 42] that FK506 has a potential use in the characterization of cellular signalling pathways at the relatively poorly defined membrane-to-nucleus stage of this process. In particular, this agent could be used to determine elements in the Ca^{2+}-mediated signalling pathways independent of activation of Ca^{2+}-dependent PKC isoenzymes.

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