Partial inhibition of human neutrophil activation by FK-506 at supratherapeutic concentrations

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Summary. The macrolide, FK-506, is a potent and effective inhibitor of lymphocyte activation. We studied the effects of FK-506 on human neutrophil activation induced by chemoattractants and by various substances which circumvent receptor stimulation. After preincubation for 5 min at 37°C, FK-506 (1 μM) inhibited N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe)- or platelet-activating factor-induced superoxide production in neutrophils by about 30%. At therapeutic concentrations (0.1–1 nM) FK-506 was ineffective. FK-506 did not inhibit exocytosis and rises in cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ mediated by these stimuli, and it did not at all inhibit neutrophil activation induced by C5a, leukotriene B_4 and 4β-phorbol 12-myristate 13-acetate. FK-506 (1 μM) inhibited A23187-induced exocytosis by about 35%, but A23187-induced superoxide production was unaffected. After preincubation for 5 min at 37°C, FK-506 inhibited fMet-Leu-Phe-induced superoxide production in dibutyryl cAMP-differentiated HL-60 cells by about 20%; preincubation with the drug for 24 h did not result in inhibition of superoxide production. FK-506 did not inhibit agonist-binding to formyl peptide receptors and fMet-Leu-Phe-stimulated GTP hydrolysis of heterotrimeric regulatory guanine nucleotide-binding proteins (G-proteins) in membranes from dibutyryl cAMP-differentiated HL-60 cells. FK-506 did not change steady-state and differential polarized phase fluorescence in HL-60 membranes using 1,6-diphenylhexa-1,3,5-triene and 12-(9-anthroyloxy)-stearate as probes. Our results show that FK-506 at supratherapeutic concentrations partially inhibits neutrophil activation. Inhibition by FK-506 of fMet-Leu-Phe-induced superoxide production is rapid in onset and is not due to inhibition of agonist-binding to receptors, interference with G-proteins or protein kinase C, reduction of rises in $[Ca^{2+}]_i$ or alteration in physical membrane state.

Introduction

Human neutrophils play an important role in non-specific host defense against bacterial infections (Malech and Gallin 1987). In neutrophils and differentiated HL-60 cells, receptors for platelet activating factor (PAF), complement C5a, leukotriene B_4 and N-formyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe), couple to heterotrimeric regulatory guanine nucleotide-binding proteins (G-proteins) (Rossi 1986; Sandborg and Smolen 1988; Dillon et al. 1988; Seifert and Schultz 1991). Activation of G-proteins results in phosphoinositide degradation with subsequent activation of protein kinase C, increase in cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$, activation of superoxide (O_2^-) production and exocytosis (Rossi 1986; Sandborg and Smolen 1988; Dillon et al. 1988; Seifert and Schultz 1991).

FK-506 is an immunosuppressant macrolide from *Streptomyces tsukabaensis* (Kino et al. 1987). The in vivo and in vitro effects of FK-506 and cyclosporin A (CsA) are similar, although there are substantial differences in potency and chemical structure of the immunosuppressants (Sigal and Dumont 1992). Similar to CsA, FK-506 inhibits mitogenic or antigenic stimulation of T- and B-lymphocytes at early stages (Wicker et al. 1990; Chang et al. 1991; Sigal and Dumont 1992). In addition, FK-506 and CsA interfere with signal transduction pathways associated with rises in $[Ca^{2+}]_i$ (Lin et al. 1991). However, inhibition by FK-506 and CsA of interleukin-2 synthesis is not associated with a reduction in $[Ca^{2+}]_i$ (Metcalfe 1984; Ryffel 1989; Bierer et al. 1990). The intracellular binding proteins for CsA and FK-506, cyclophilin and FK-506-binding protein (FKBP), respectively, do not share amino acid sequence homology (Siekierka et al. 1989; Harding et al. 1989), but both immunosuppressant-immunophilin-complexes inhibit the

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Ca^{2+}-/calmodulin-dependent phophatase, calcineurin (Liu et al. 1991).

At therapeutic concentrations CsA only marginally inhibits human neutrophil activation (Janco and English 1983; Kharazmi et al. 1985; Wenzel-Seifert et al. 1991). Interestingly, FK-506 has recently been reported to be a very potent and effective inhibitor of A 23187-induced exocytosis in human neutrophils (Forrest et al. 1991). Additionally, FK-506 partially inhibits interleukin-1α release from human macrophages (Keicho et al. 1991) and potently inhibits mediator release from basophils and mast cells (De Paulis et al. 1991a, b; Hultsch et al. 1991). All these findings prompted us to study in detail the effects of FK-506 on human neutrophil activation. We show here that FK-506 partially inhibits O_2^- production and exocytosis at supratherapeutic concentrations.

Materials and methods

Materials. FK-506 was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Stock solutions of FK-506 (1 mM) were prepared in dimethyl sulfoxide and were stored in polypropylene tubes at −20°C under light protection for up to two months. Dilutions of FK-506 were made immediately prior to experiments. The final concentration of dimethyl sulfoxide in all experiments was adjusted to 0.1% (v/v). 12-(9-Anthroyloxy)-stearate (12-AS) was obtained from Molecular Probes (Junction City, Ore., USA); 1,6-diphenylhexa-1,3,5-triene (DPH) was from EGA Chemie (Steinheim, FRG). Stock solutions of DPH (2 mM) were prepared in tetrahydrofurane and were kept at 25°C. Stock solutions of 12-AS (1 mM) were prepared in 80% ethanol (v/v) and were kept at −20°C. Sources of other materials have been described elsewhere (Seifert et al. 1989a,b, 1992; Wenzel-Seifert and Seifert 1990; Wenzel-Seifert et al. 1991).

Isolation of human neutrophils. Neutrophils were obtained from 12 caucasian healthy volunteers (six males and six females) approximately 2 h after a continental breakfast. Volunteers had taken no drugs for at least three weeks. The age of volunteers ranged from 28–40 years; six of them were smokers. Isolation of neutrophils was performed as described (Seifert et al. 1989a). Cell preparations consisted of >95% viable neutrophils as revealed by trypan blue dye exclusion and Pappenheim-stained smears.

Assay for O_2^- production. O_2^- production was monitored at 550 nm by continuous measurement of ferricytochrome C reduction inhibitable by superoxide dismutase, using an Uvikon 810 dual beam spectrophotometer (Kontron, Eching, FRG) (Seifert et al. 1989a). Reaction mixtures (0.5 ml) contained 100 µM ferricytochrome C and a buffer consisting of (mM) 138 NaCl, 6 KCl, 1 MgCl₂, 1 CaCl₂, 5.5 glucose and 20 Hepes/NaOH, pH 7.4. Neutrophils (1.0–2.0×10⁶ cells) or dibutyryl cAMP-differentiated HL-60 cells (2.5×10⁶ cells) were suspended in the solution described above and were incubated for 5 min at 37°C in the presence of FK-506 or solvent. O_2^- production was initiated by the addition of stimuli.

Assay for β-glucuronidase and lysozyme release. Exocytosis was assessed as described (Seifert et al. 1989b; Wenzel-Seifert and Seifert 1990; Wenzel-Seifert et al. 1991). In one set of experiments, neutrophils (4.0–5.0×10⁶ cells) were incubated for 5 min at 37°C in the buffer (0.5 ml) described above supplemented with cytochalasin B (5 µg/ml) in the presence of FK-506 or solvent. Thereafter, stimuli were added to cells. In another set of experiments, neutrophils (4.0–5.0×10⁶ cells) were incubated for 25 min at 20°C in the buffer (0.5 ml) described above not supplemented with cytochalasin B, in the presence of FK-506 or solvent. Thereafter, cells were incubated for additional 5 min at 37°C in the presence of FK-506 or solvent plus cytochalasin B (5 µg/ml). Subsequently, stimuli were added to cells. Reactions were terminated after

10 min by rapid cooling to 4°C. Reaction mixtures were centrifuged for 10 min at 1000×g. The determinations of the activities of β-glucuronidase, lysozyme and lactate dehydrogenase of the supernatant fluids of reaction mixtures and of cell lysates were performed as described (Seifert et al. 1989b; Wenzel-Seifert and Seifert 1990; Wenzel-Seifert et al. 1991). Basal β-glucuronidase, lysozyme and lactate dehydrogenase release amounted to less than 5% of cellular content (data not shown).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined using the fluorescent dye, Fura-2, as described (Wenzel-Seifert and Seifert 1990). Neutrophils were suspended at 5×10⁶ cells/ml in a buffer consisting of (mM) 138 NaCl, 6 KCl, 1 MgSO₄, 1.1 CaCl₂, 0.1 EGTA, 1 Na₂HPO₄, 5 NaHCO₃, 5.5 glucose, 20 Hepes/NaOH, pH 7.4, supplemented with bovine serum albumin (0.1%, w/v). Cells were incubated for 1 h at 37°C in the presence of Fura-2 acetoxymethylester (4 µM). Subsequently, cells were diluted with the above buffer to a final concentration of 0.5×10⁶ cells/ml and were centrifuged for 10 min at 250×g at 20°C. Cells were suspended at 2.5×10⁶ cells/ml in the above buffer and were kept at 20°C until measurement of $[Ca^{2+}]_i$. Fluorescence of neutrophils (2.5×10⁶ cells in 2 ml) was determined at 37°C under constant stirring at 10³ rpm using a Ratio II spectrofluorometer (Aminco, Silver Spring, Md., USA). Cells were incubated for 5 min in the presence of FK-506 or solvent prior to the addition of stimuli. The excitation and emission wavelengths were 340 and 500 nm, respectively.

Cell culture and membrane preparation. HL-60 cells were cultured in suspension culture at 37°C and were differentiated towards neutrophil-like cells upon incubation with dibutyryl cAMP (0.2 mM) for 48 h (Seifert et al. 1989a; Wenzel-Seifert and Seifert 1990). In some experiments, FK-506 or solvent were added to cultures 24 h prior to experiments. HL-60 membranes were prepared as described (Seifert and Schultz 1987).

Steady-state and differential polarized phase fluorometry. DPH and 12-AS were added from stock solutions to fluorometry buffer consisting of (mM) 100 mannitol, 10 Hepes/Tris, pH 7.4, and were stirred at 25°C for 1 h and 5 min, respectively, to remove solvent. HL-60 membranes were suspended in these solutions to give final probe concentrations of 1 µM (DPH) and 2 µM (12-AS); these mixtures were incubated in the dark for 30 min at 37°C. Membranes were centrifuged for 20 min at 48000×g at 4°C and were resuspended in fluorometry buffer at 0.15 mg of protein/ml. Membranes were incubated for 10 min at 25°C in the presence of FK-506 or solvent. Fluorescence measurements were performed at 25°C in a SLM 4800 spectrofluorometer (SLM Instruments, Urbana, Ill. USA) equipped with a thermostated cuvette holder. Steady state-anisotropy was measured on the T formate with 360 nm (DPH) and 365 nm (12-AS) as excitation wavelengths. Emission light was filtered through KV-418 filters (Schott, Mainz, FRG). Fluorescence lifetimes were measured at 18 MHz by the phase modulation technique relative to a glycogen scatter solution (Spencer and Weber 1969). Differential tangent was measured by differential polarized phase fluorometry at 18 MHz (Lakowicz et al. 1979). Rotational correlation time and limiting anisotropy were calculated from steady-state anisotropy, phase lifetime, differential tangent and r_0, i.e. the anisotropy in the absence of motion (Lakowicz et al. 1979). R_0 values of 0.390 (DPH) (Lakowicz et al. 1979) and 0.285 (12-AS) (Schachter and Shinitzky 1977) were used for calculations.

fMet-Leu-[³H]Phe binding assay. fMet-Leu-[³H]Phe binding was performed as described (Wenzel-Seifert et al. 1991). Reaction mixtures (100 µl) contained 40 µg of membrane protein from dibutyryl cAMP-differentiated HL-60 cells, bovine serum albumin (0.2%, w/v), 50 mM Tris/HCl, pH 7.3, 1 mM EDTA and 5 mM MgCl₂ in the presence of FK-506 or solvent. Incubations were conducted for 30 min at 25°C and were initiated by the addition of 3 nM fMet-Leu-[³H]Phe. Reactions were terminated by rapid filtration technique. Radioactivity on dried filters was determined in a liquid scintillation counter. Unspecific binding was determined in the presence of 10 µM fMet-Leu-Phe and amounted to less than 10% of total binding.

Measurement of GTPase activity. GTPase activity was measured as described (Seifert et al. 1992). Assay mixtures (100 μl) contained membranes from HL-60 cells (7.0 μg of protein/tube), 0.5 μM [γ-^{32}P]GTP (0.1 μCi/tube), 0.5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 1 mM adenosine 5'-[β, γ-imido]-triphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, 1 mM dithiothreitol and 0.2% (w/v) bovine serum albumin in 50 mM triethanolamine/HCl, pH 7.4. Reaction mixtures were preincubated for 10 min at 25 °C with FK-506 or solvent. Thereafter, fMet-Leu-Phe or H_2O were added. After additional 10 min at 25 °C, reactions were initiated by the addition of [γ-^{32}P]GTP and were conducted for 15 min. Low-affinity GTPase activity was determined in the presence of GTP (50 μM) and amounted to <5% of total GTPase activity.
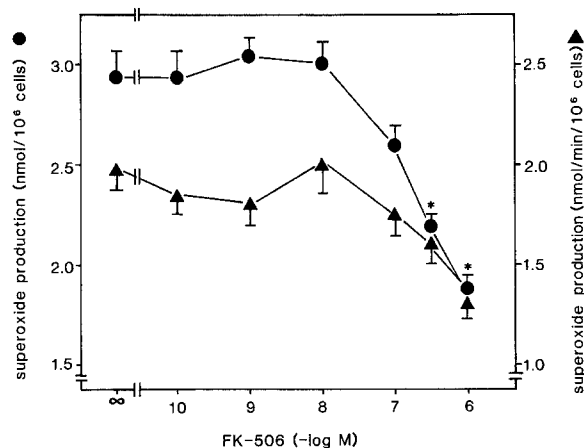
Miscellaneous. Protein was determined according to Lowry et al. (1951). The effects of FK-506 were statistically assessed using the Wilcoxon test.
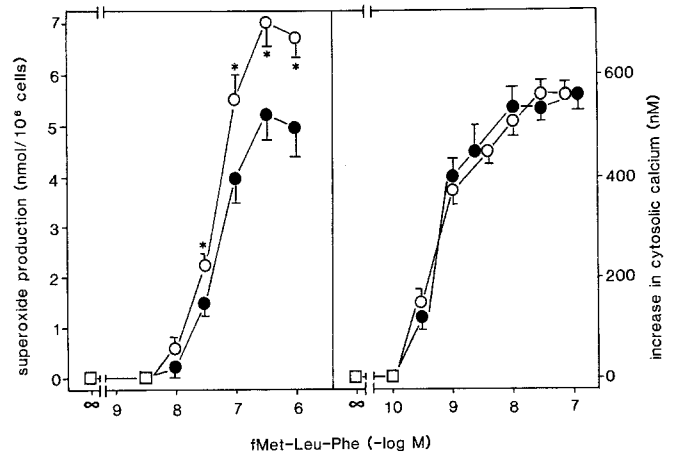
Results

First, the effects of FK-506 on cell integrity were studied. FK-506 up to 1 μM did not cause damage of neutrophils and HL-60 cells as revealed by lactate dehydrogenase release and trypan blue dye exclusion (data not shown).

The effect of FK-506 at various concentrations on O_2^- production induced by fMet-Leu-Phe (50 nM) is shown in Fig. 1. FK-506 above 100 nM inhibited the absolute amount and maximum rate of O_2^- production by up to 35%. At lower concentrations, FK-506 was ineffective.

The effects of FK-506 (1 μM) on O_2^- production and on rises in [Ca^{2+}]$_i$ induced by various stimuli were studied. fMet-Leu-Phe activated O_2^- production with an EC_{50} of 45 nM and a maximum at 300 nM (Fig. 2). FK-506 reduced O_2^- production by about 30% at all fMet-Leu-Phe concentrations employed, and it did not substantially increase the EC_{50}. fMet-Leu-Phe increased [Ca^{2+}]$_i$ with an EC_{50} of 0.4 nM and a maximum at 30 nM (see Fig. 2); FK-506 was without effect on rises in

Fig. 2. Effect of FK-506 on concentration-response functions to fMet-Leu-Phe-induced O_2^- production and rises in [Ca^{2+}]$_i$ in human neutrophils. FK-506 (1 μM) (●) or solvent (control) (○) were added to reaction mixtures 5 min prior to fMet-Leu-Phe at various concentrations. *Open squares* show that fMet-Leu-Phe at the indicated concentrations did not activate O_2^- production or increase [Ca^{2+}]$_i$. Data shown are the means±SEM of assay triplicates of four experiments carried out with neutrophils from different donors. Comparison of the effect of FK-506 versus control, *$P < 0.05$

[Ca^{2+}]$_i$. PAF activated O_2^- production and increased [Ca^{2+}]$_i$ with EC_{50} values of 300 nM and 5 nM, respectively (Fig. 3). Similar to fMet-Leu-Phe, FK-506 inhibited PAF-induced O_2^- production by about 30%, but it did not affect rises in [Ca^{2+}]$_i$. FK-506 did not inhibit O_2^- production induced by C5a (1 and 10 nM), leukotriene B$_4$ (1 μM), A 23187 (10 μM) and 4β-phorbol 12-myristate 13-acetate (PMA) (10, 30 and 100 ng/ml) and increases in [Ca^{2+}]$_i$ induced by C5a (0.01 – 3 nM) and leukotriene B$_4$ (0.3 – 100 nM) (data not shown). FK-506 by itself did not induce a rise in [Ca^{2+}]$_i$ and did not activate O_2^- production (data not shown).
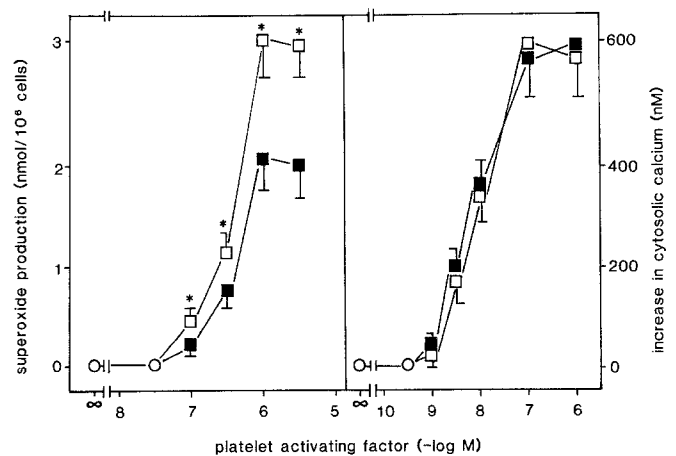
Fig. 1. Concentration-response function to FK-506 of fMet-Leu-Phe-induced O_2^- production in human neutrophils. The effect of FK-506 on the absolute amount and maximum rate of O_2^- production induced by fMet-Leu-Phe (50 nM) was studied. FK-506 or solvent (control) were added to reaction mixtures 5 min prior to fMet-Leu-Phe. Data shown are the means±SEM of assay triplicates of five experiments carried out with neutrophils from different donors. Comparison of the effect of FK-506 versus control, *$P < 0.05$

Fig. 3. Effect of FK-506 on concentration-response functions to PAF-induced O_2^- production and rises in [Ca^{2+}]$_i$ in human neutrophils. FK-506 (1 μM) (■) or solvent (control) (□) were added to reaction mixtures 5 min prior to PAF at various concentrations. *Open circles* show that PAF at the indicated concentrations did not activate O_2^- production or increase [Ca^{2+}]$_i$. Data shown are the means±SEM of assay triplicates of five experiments carried out with neutrophils from different donors. Comparison of the effect of FK-506 versus control, *$P < 0.05$

Table 1. Effect of FK-506 on β-glucuronidase and lysozyme release in human neutrophils

Addition	Enzyme release (% of cellular content)			
	Preincubation			
	25 min (20 °C) + 5 min (37 °C)		5 min (37 °C)	
	β-Glucuronidase	Lysozyme	β-Glucuronidase	Lysozyme
A23187 (5 µM) + solvent	31 ± 2	47 ± 4	24 ± 2	47 ± 5
A23187 (5 µM) + FK-506	19 ± 1 [c]	31 ± 4 [b]	15 ± 1 [c]	36 ± 5 [b]
fMet-Leu-Phe (30 nM) + solvent	10 ± 1	19 ± 2	12 ± 2	13 ± 1
fMet-Leu-Phe (30 nM) + FK-506	10 ± 1 [a]	19 ± 2 [a]	11 ± 1 [a]	13 ± 2 [a]
PAF (1 µM) + solvent	17 ± 1	17 ± 3	18 ± 2	18 ± 2
PAF (1 µM) + FK-506	14 ± 2 [a]	16 ± 3 [a]	15 ± 1 [a]	15 ± 1 [a]

Neutrophils were preincubated with FK-506 (1 µM) or solvent (control) under various conditions. Release of β-glucuronidase and lysozyme induced by various stimuli was assessed. Data shown are the means ± SEM of assay triplicates of four experiments carried out with neutrophils from different donors
Comparison of the effect of FK-506 versus control
[a] Not significant
[b] $P < 0.05$
[c] $P < 0.01$

Table 2. Effect of FK-506 on O_2^- production in dibutyryl cAMP-differentiated HL-60 cells

Addition	O_2^- production (nmol/10^6 cells)	
	Preincubation	
	5 min (37 °C)	24 h (37 °C)
Solvent	2.1 ± 0.2	2.0 ± 0.2
FK-506	1.5 ± 0.2 [b]	2.2 ± 0.2 [a]

In one set of experiments, HL-60 cells were harvested and preincubated for 5 min with FK-506 (1 µM) or solvent (control) prior to the addition of fMet-Leu-Phe (1 µM). In another set of experiments, FK-506 (1 µM) or solvent (control) were added to cell cultures 24 h prior to experiments. Subsequently, cells were harvested and O_2^- production induced by fMet-Leu-Phe (1 µM) was assessed. Data shown are the means ± SEM of assay triplicates of four experiments with different preparations of HL-60 cells
Comparison of the effect of FK-506 versus control
[a] Not significant
[b] $P < 0.05$

The effects of FK-506 on exocytosis are shown in Table 1. FK-506 (1 µM) inhibited A 23187-induced β-glucuronidase and lysozyme release by 30–40%, regardless of whether cells were incubated with the drug for 25 min at 20 °C plus 5 min at 37 °C or only for 5 min at 37 °C. FK-506 did not inhibit fMet-Leu-Phe- and PAFF-induced exocytosis. FK-506 per se did not activate exocytosis (data not shown).

Modulation by FK-506 of O_2^- production in dibutyryl cAMP-differentiated HL-60 cells is shown in Table 2. After preincubation for 5 min at 37 °C FK-506 inhibited fMet-Leu-Phe-induced O_2^- production by about 20%. By comparison, fMet-Leu-Phe-induced O_2^- production was not altered in cells preincubated with FK-506 for 24 h at 37 °C.

The effect of FK-506 on binding of fMet-Leu-[^{3}H]Phe (3 nM) to formyl peptide receptors in membranes from dibutyryl cAMP-differentiated HL-60 cells

was investigated. Agonist-binding in control membranes was 568 ± 27 fmol/mg protein. FK-506 (1 µM) did not alter agonist-binding to formyl peptide receptors (569 ± 24 fmol/mg protein).

Modulation by FK-506 of high-affinity GTPase activity in membranes from dibutyryl cAMP-differentiated HL-60 cells was studied. Basal GTP hydrolysis was 17.1 ± 1.5 pmol P_i/mg protein/min. fMet-Leu-Phe (0.3 µM) stimulated GTP hydrolysis by 80%. FK-506 (1 µM) showed no effect on basal and fMet-Leu-Phe-stimulated GTP hydrolysis (data not shown).

Finally, the effect of FK-506 on physical state of HL-60 membranes was studied. Steady-state anisotropy in control membranes was 0.2109 ± 0.0033 with DPH as probe. Phase lifetime of DPH was 9.47 ± 0.07 ns. Limiting anisotropy was 0.180 ± 0.002 and rotational correlation time was 1.62 ± 0.11 ns. FK-506 (1 µM) had no effect on these parameters (data not shown). Steady-state anisotropy and phase lifetime of 12-AS in control membranes were 0.0849 ± 0.0015 and 11.12 ± 0.10 ns, respectively; FK-506 (1 µM) showed no effect on these parameters. FK-506 did not change limiting anisotropy and rotational correlation time of 12-AS in HL-60 membranes (data not shown).

Discussion

We found that FK-506 partially inhibited fMet-Leu-Phe- and PAF-induced O_2^- production and A 23187-induced exocytosis in human neutrophils at a supratherapeutic concentration (1 µM) (see Figs. 2 and 3 and Table 1) (Starzl et al. 1989). Additionally, FK-506 (1 µM) slightly inhibited fMet-Leu-Phe-induced O_2^- production in differentiated HL-60 cells (see Table 2). These findings prompted us to study the effects of FK-506 at therapeutic concentrations on fMet-Leu-Phe-induced O_2^- production in human neutrophils as fMet-Leu-Phe is assumed to play a key role in neutrophil activation in bacterial infec-

tions (see Fig. 1) (Rossi 1986; Sandborg and Smolen 1988; Seifert and Schultz 1991). However, at therapeutic concentrations (0.1 – 1 nM) (Starzl et al. 1989), FK-506 did not inhibit O_2^- production induced by fMet-Leu-Phe (see Fig. 1). Additionally, FK-506 was without effect on O_2^- formation induced by leukotriene B_4 and C5a, two other physiologically relevant chemoattractants (Rossi 1986; Sandborg and Smolen 1988; Seifert and Schultz 1991). Furthermore, FK-506 did not at all affect exocytosis induced by fMet-Leu-Phe and PAF (see Table 1). These data suggest that the only partial inhibition of neutrophil activation by FK-506 at supratherapeutic concentrations is without clinical relevance and that FK-506 at therapeutic concentrations is unlikely to impair the non-specific defense system substantially.

The effects of FK-506 on neutrophil activation showed some similarities with the corresponding effects of CsA. The effectiveness of CsA and FK-506 (1 μM each) to inhibit fMet-Leu-Phe-induced O_2^- production is similar (see Figs. 1 and 2) (Wenzel-Seifert et al. 1991). Like CsA, FK-506 did not inhibit fMet-Leu-Phe- and PAF-induced exocytosis and rises in $[Ca^{2+}]_i$ (see Figs. 2 and 3 and Table 1) (Wenzel-Seifert et al. 1991). Similar to CsA, FK-506 did not interfere with agonist-binding to formyl peptide receptors (Wenzel-Seifert et al. 1991). However, unlike CsA, FK-506 partially inhibited PAF-induced O_2^- production (see Fig. 3) (Wenzel-Seifert et al. 1991). These data suggest that FK-506 and CsA inhibit neutrophil activation through related but not identical mechanisms.

FKBP is ubiquitously distributed in eukaryotic cells (Siekierka et al. 1989). In human lymphocytes, the potency of FK-506 in functional assays (IC_{50} approximately 0.1 nM) is in accordance with the affinity of FK-506 to FKBP (K_D) of 0.4 – 0.8 nM) (Sigal and Dumont 1992; Siekierka et al. 1989). These data suggest that the FK-506-FKBP complex mediates the immunosuppressive effects of the drug in these cells. In addition, FK-506 inhibits activation of mast cells and basophils with IC_{50} values of 1 – 5 nM (De Paulis et al. 1991 a, b; Hultsch et al. 1991). By comparison, FK-506 at these concentrations was without effect on fMet-Leu-Phe-induced O_2^- production, and FK-506 (1 μM) showed no or only relatively small inhibitory effects on various neutrophil activation parameters (see Figs. 1 – 3 and Tables 1 and 2). These data suggest that the inhibitory effects of FK-506 on neutrophil activation are not mediated by the FK-506-FKBP complex. It cannot, however, be excluded that FK-506 penetrates the neutrophil plasma membrane only poorly, i.e. the drug has to be present at very high extracellular concentrations to reach sufficient concentrations in the cytosol for interaction with FKBP.

Several other possibilities have to be discussed to explain the inhibitory effects of FK-506 on human neutrophil activation. First, the failure of FK-506 to inhibit agonist-binding to formyl peptide receptors suggests that it does not act as formyl peptide receptor antagonist. Second, the lack of effect of FK-506 on high-affinity GTPase in HL-60 membranes indicates that the drug does not interfere with G-proteins. Third, FK-506 did not inhibit PMA-induced O_2^- production suggesting that it does not

act as inhibitor of protein kinase C. Fourth, the lack of effect of FK-506 on O_2^- production induced by various stimuli implies that it does not interfere with a component of NADPH oxidase, the O_2^--generating enzyme system (Rossi 1986; Seifert and Schultz 1991). Similarly, the failure of FK-506 to inhibit exocytosis by various stimuli implies that it does not interfere with secretory mechanisms in an unspecific manner (see Table 1). Additionally, the lack of effect of FK-506 on fMet-Leu-Phe- and PAF-induced rises in $[Ca^{2+}]_i$ indicates that the drug does not interfere with Ca^{2+} mobilization and/or Ca^{2+} influx from the extracellular space (see Figs. 2 and 3). Furthermore, we did not observe toxic effects of FK-506 on human neutrophils. Finally, the inhibitory effects of FK-506 were evident after short-term preincubation of cells but not after long-term treatment (see Figs. 1 – 3 and Tables 1 and 2). Thus, FK-506 apparently does not act via inhibition of gene transcription or protein synthesis. Interestingly, inhibitory effects of FK-506 on basophil and mast cell activation are also evident after short-term preincubation (De Paulis et al. 1991 a, b; Hultsch et al. 1991).

FK-506 is a very lipophilic drug (Kino et al. 1987). This fact and the supratherapeutic concentrations of FK-506 required to partially inhibit neutrophil activation (see Fig. 1) (Starzl et al. 1989) raise the question of whether the drug acts via alteration of physical membrane state. A widely employed technique for the study of physical membrane state is steady-state polarization and differential polarized phase fluorometry with DPH and 12-AS as probes. Absolute steady-state anisotropy of DPH in dibutyryl cAMP-differentiated HL-60 membranes was similar to the one in membranes from dimethyl sulfoxide-differentiated HL-60 cells, human neutrophils, lymphocytes and erythrocytes (Shinitzky and Inbar 1974; Ip and Cooper 1980; Giraud et al. 1991; Yuli et al. 1982). Limiting anisotropy reflects lipid packing order in the membrane restricting the probes' rotational motion, i.e. it is a static measure; rotational correlation time reflects mobility of the probe in the lipid environment, i.e. it is a dynamic measure (Lakowicz et al. 1979). The lack of effect of FK-506 on limiting anisotropy and rotational correlation time clearly argues against alterations in physical membrane state accounting for its partial inhibitory effects on neutrophil activation. Taken together, the mechanism of action of FK-506 in human neutrophils is still unknown.

There are some similarities and dissimilarities between the results obtained by us und by Forrest et al. (1991). In agreement with our data are their findings that FK-506 and CsA do not inhibit fMet-Leu-Phe-induced exocytosis (see Table 1) (Wenzel-Seifert et al. 1991). However, Forrest et al. (1991) reported that FK-506, after preincubation for 25 min at room temperature, inhibited exocytosis induced by A 23187 at a maximally effective concentration (1 μM) in human neutrophils with an IC_{50} of about 1 nM and a maximum at 10 nM. In our hands, A 23187 was maximally effective at 5 μM, whereas A 23187 at 1 μM induced the release of only about 5% of the cellular content of β-glucuronidase release, i.e. this stimulation was too small for reliable quantitative assessment of the inhibitory effect of FK-506 (data not shown).

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Differences in the maximally effective concentrations of A 23187 for stimulation of exocytosis among various groups have been reported before (Kang et al. 1985; Becker et al. 1985; Naccache et al. 1985). In contrast to Forrest et al. (1991), we found that FK-506 as high as 1 µM showed only relatively small inhibitory effects on A 23187-induced exocytosis, regardless of whether cells were preincubated with the drug for 25 min at 20 °C plus 5 min at 37 °C or only for 5 min at 37 °C (see Table 1). Basically, the methods applied by us and by Forrest et al. (1991) (see Materials and methods) were similar. It is possible that differences in responsiveness to FK-506 of neutrophils in both studies existed. Interestingly, Kahan et al. (1991) reported on substantial variation in responsiveness to FK-506 among lymphocytes from different donors. In addition, it is known that regulation of O_2^- production may depend on several conditions such as sex, age, active and passive smoking, feeding state and method of cell isolation (Seifert and Schultz 1991; Lipschitz et al. 1991; Anderson et al. 1991; Uhlinger et al. 1991). With regard to the donors studied by us, however, FK-506 showed very consistent effects (see Figs. 1–3 and Table 1). Specifically, we did not observe that neutrophils of some donors were highly sensitive to inhibition by FK-506, whereas cells from other donors were insensitive. Unfortunately, Forrest et al. (1991) did not provide sufficient information on the above-mentioned factors in their volunteers. Regardless of the reason for the differences between our results and those of Forrest et al. (1991) should it be kept in mind that A 23187, unlike fMet-Leu-Phe, C5a, PAF and leukotriene B_4, is not a physiologically relevant stimulus (Rossi 1986; Sandborg and Smolen 1988; Seifert and Schultz 1991).

In conclusion, we have shown that FK-506 at therapeutic concentrations does not inhibit human neutrophil activation. At supratherapeutic concentrations FK-506 partially inhibits neutrophil activation through a mechanism which is rapid in onset and does not involve inhibition of agonist binding to formyl peptide receptors, interference with G-proteins or protein kinase C, reduction of rises in $[Ca^{2+}]_i$ or changes in physical membrane state.

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