

Effects of rapamycin, cyclosporin A, and dexamethasone on interleukin 5-induced eosinophil degranulation and prolonged survival

Meng Q, Ying S, Corrigan CJ, Wakelin M, Assoufi B, Moqbel R, Kay AB. Effects of rapamycin, cyclosporin A, and dexamethasone on interleukin 5-induced eosinophil degranulation and prolonged survival. *Allergy* 1997; 52: 1095–1101. © Munksgaard 1997.

Interleukin-5 (IL-5) enhances eosinophil degranulation and prolongs eosinophil survival. Rapamycin, cyclosporin A, and dexamethasone have been shown to influence either cytokine transcription, cytokine-mediated signalling, or degranulation by granulocytes. The study aimed to determine whether these agents inhibited IL-5-enhanced eosinophil survival or degranulation. Peripheral blood eosinophils were isolated from atopic subjects. The effects of serial dilutions (10^{-6} – 10^{-9} M) of these drugs or vehicle control on 1) the viability of eosinophils cultured (1–5 days) in the presence and absence of recombinant human IL-5, as measured by propidium iodide staining and flow cytometry, and 2) degranulation of eosinophils preincubated (45 min) with rhIL-5 or medium control, as measured by eosinophil cationic protein (ECP) release after stimulation with serum-coated Sephadex beads, were assessed. Dexamethasone and rapamycin produced significant, concentration-dependent inhibition of IL-5-enhanced eosinophil survival at pharmacologic concentrations, whereas cyclosporin A did not. Prior incubation of eosinophils with IL-5, as compared with medium control, significantly enhanced ECP release by eosinophils on subsequent exposure to serum-coated Sephadex beads. Cyclosporin A and rapamycin significantly inhibited IL-5-enhanced ECP release in a concentration-dependent fashion, whereas dexamethasone did not. All three drugs had no significant effect on eosinophil survival and degranulation in the absence of IL-5. Our results suggest that immunosuppressive drugs may inhibit IL-5-mediated mechanisms in eosinophils which result in enhanced survival and release of granule contents. These findings may be relevant to the further development of therapeutic strategies in allergic diseases.

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Key words: cyclosporin A; degranulation; dexamethasone; eosinophil; interleukin-5; rapamycin; survival.

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Accepted for publication 19 June 1997

Eosinophils are believed to play a major role in the pathogenesis of asthma and other disorders associated with chronic allergic inflammation through secretion of preformed and newly synthesized basic proteins and lipid mediators (1). Thus, measures which inhibit the ingress, activation, survival, and secretion of eosinophils at the site of allergic tissue reactions may be of benefit in the treatment of asthma and allied disorders.

Asthmatic inflammation is believed to be orchestrated, at least partly, by release of eosinophil-active cytokines (interleukin-3 [IL-3], IL-5, and granulocyte/macrophage colony-stimulating factor

[GM-CSF]) from T cells. Whereas all of these cytokines enhance eosinophil differentiation, activation, and survival, IL-5 is unique in acting specifically on mature eosinophils and their committed precursors (2). In addition, IL-5 has been shown to delay glucocorticoid-mediated apoptosis of eosinophils (3, 4) and enhance eosinophil granule release, adherence, and phagocytosis (5). Furthermore, IL-5 synthesis is elevated in peripheral blood, and bronchial, mucosal, and bronchoalveolar lavage (BAL) fluid T cells of asthmatics (6–8), and IL-5 is the only cytokine the expression of which consistently correlates with disease severity and

changes in severity after glucocorticoid therapy (6–9).

Since glucocorticoids appear to ameliorate asthma, possibly by inhibiting T-cell activation and synthesis of cytokines such as IL-5, there has been recent interest in other drugs which inhibit T-cell function for asthma therapy. This is especially important in those patients who continue to have severe disease despite high dosages of oral glucocorticoids, which have unwanted side-effects. In a placebo-controlled trial, the immunosuppressive drug cyclosporin A was shown to improve lung function in asthma and to reduce the frequency of disease exacerbation after 12 weeks of therapy (10). Although both cyclosporin A and the macrolide rapamycin inhibit T-cell proliferation, their mechanisms of action appear to be fundamentally different (11–13). Cyclosporin A inhibits early T-cell activation events after ligation of the T-cell receptor, resulting in reduced translocation of the cytoplasmic subunit of the T-cell IL-2 transcriptional activator NF-AT to the nucleus. It is effective only when present early after T-cell activation and does not inhibit IL-2/IL-4-mediated T-cell division once initiated. Rapamycin, on the other hand, inhibits IL-2-mediated T-cell proliferation, even in T cells committed to mitosis. It probably acts by inhibiting phosphorylation/activation of the kinase p70 S6 (p70^{s6k}), and by inhibiting the enzymatic activity of the cyclin-dependent kinase cdk2-cyclin E complex. Thus, rapamycin impedes progression through the G1/S transition of the proliferation cycle, resulting in a mid-to-late G1 arrest (14). Therefore, we hypothesized that rapamycin may inhibit cytokine-mediated activation processes in other cell types.

On the other hand, the actions of cyclosporin A do not appear to be explicable solely in terms of NF-AT modulation, since this drug rapidly inhibits degranulation of basophils (15), an effect which may reflect its inhibition of immunophilin topoisomerase activity. In addition, cyclosporin A inhibits release of GM-CSF from mature eosinophils (16), an effect which may depend at least partly on inhibition of degranulation as well as *de novo* cytokine synthesis.

For all these reasons, we hypothesized that dexamethasone, cyclosporin A and rapamycin inhibit IL-5-enhanced eosinophil survival and degranulation. We aimed to examine the effects of these drugs on these two aspects of eosinophil function in the presence and absence of IL-5 *in vitro*.

Material and methods

Reagents and drugs

Recombinant human IL-5 (rhIL-5) was purchased from Genzyme (Cambridge, MA, USA). Rapamycin

(Rapa), cyclosporin A (CsA), and dexamethasone (Dex) were obtained from Wyeth-Ayerst (Princeton, NJ, USA), Sandoz Pharma (Basel, Switzerland), and Sigma Chemical Company (Poole, UK), respectively. Rapa and CsA were dissolved in ethanol at a concentration of 10^{-4} mol/l and stored in glass vials in aliquots. Dex was dissolved directly in RPMI-1640 at a concentration of 10^{-4} mol/l. In use, serial 10-fold dilution of drugs were made at the time of each experiment by dissolving the stock solutions in RPMI-1640 medium (Gibco, Paisley, UK) containing 2 mM L-glutamine. Vehicle control solutions were prepared by dissolving vehicle in medium to produce final concentrations of vehicle equivalent to those in the highest concentrations of the drugs employed.

Eosinophil purification

Eosinophils were purified from peripheral blood as previously described (17), with some modifications. Briefly, heparinized human peripheral blood obtained from subjects with mild asthma was mixed with $0.2 \times$ volume of 6% dextran, and the erythrocytes were allowed to sediment for 40 min at room temperature. The upper layer (leukocyte-rich plasma) was collected, layered onto Histopaque (relative density 1.083, Sigma, UK), and centrifuged at 1500 rpm for 25 min. Eosinophils were isolated and purified from the resulting granulocyte pellet by negative immunomagnetic selection with anti-CD16-coated Microbeads and the MACS system (Becton Dickinson, Cowley, UK). To eliminate any contamination with mononuclear cells, anti-CD14- and anti-CD3-coated beads (Becton Dickinson) were also added to the anti-CD16 bead mixture. By negative selection, isolates consisting of >99% eosinophils, as determined by Kimura staining and confirmed by differential counts with May-Grünwald-Giemsa stain, and viabilities of >95%, as determined by trypan blue exclusion, were routinely obtained.

Eosinophil survival assay

Purified human peripheral blood eosinophils were resuspended (2×10^6 cells/ml, total volume 100 μ l) in RPMI-1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were incubated (up to 5 days) in sterile, 96-well, flat-bottomed culture plates at 37°C in a 5% CO₂ atmosphere. For determination of the effects of drugs on cytokine-induced eosinophil survival, eosinophils were cultured in the presence of rhIL-5 (final concentration 10 ng/ml), or medium control, and 10-fold serial drug dilutions (10^{-6} – 10^{-9} M) or vehicle control. After culture,

eosinophils were stained by adding 50 µl of propidium iodide (PI, 50 ng/ml, Sigma, Poole, UK) for 5 min, and then washing with cold phosphate-buffered solution (PBS). Viable cells were identified by their ability to exclude PI as analysed by flow cytometry (Coulter Epics Elite, Bedfordshire, UK). This was used to calculate survival (%) and drug-induced inhibition of IL-5-enhanced survival by the following formula:

$$\text{inhibition of survival (\%)} = \frac{(V_{\text{IL-5}} - V_{\text{IL-5+drug}})/V_{\text{IL-5}} \times 100\%}{V_{\text{IL-5}}}$$

where $V_{\text{IL-5}}$ = percent viability in the presence of rhIL-5 and drug vehicle, and $V_{\text{IL-5+drug}}$ = percent viability in the presence of rhIL-5 and drug.

Effects of drugs on eosinophil degranulation

Degranulation of eosinophils was induced with serum-coated beads. Sephadex G-15 beads (250 mg) (Sigma, UK) were treated with 3.0 ml of 50% (v/v) fresh normal human serum in $1 \times$ Hanks' balanced salt solution (HBSS), as previously described (18). Aliquots of eosinophils (150 µl, $3 \times 10^6/\text{ml}$) were then incubated (37°C , 5% CO_2) with bead suspension (300 µl), and supernatants were collected at 1, 5, 10, 15, and 30 min, and stored at -80°C until analysed. Eosinophil cationic protein (ECP) concentrations in supernatants were measured by radioimmunoassay (Pharmacia, Uppsala, Sweden; detection range 2–200 µg/l). For determination of the effects of drugs, the eosinophils were preincubated with each drug (10^{-6} – 10^{-9} M) or vehicle control for 45 min before stimulation with serum-treated beads. For determination of whether rhIL-5 enhanced ECP release induced by beads and whether the drugs inhibited this enhancement, the purified eosinophils were preincubated (45 min, 37°C) with rhIL-5 (20 ng/ml) alone, or in the presence of drugs at various concentrations, and then stimulated (30 min) with serum-coated beads. Finally, in all experiments, as a positive control for degranulation, the eosinophils were incubated (30 min) with cytochalasin B (5 mg/ml) and FMLP (10^{-6} M) (18). HBSS-treated beads were used as negative controls. All assays were performed in duplicate. Both the intra- and interassay coefficients of variation were $<5\%$. The results were expressed as percentages of the total ECP release compared with that released from freeze-thawed cells and ng/ml (mean $\pm$ SEM) of ECP released into the supernatants. The percent inhibition of rhIL-5-enhanced ECP release by the drugs was determined by the following formula:

$$\text{inhibition of release (\%)} = \frac{(\text{ECP}_{\text{IL-5}} - \text{ECP}_{\text{IL-5+drug}})/\text{ECP}_{\text{IL-5}} \times 100\%}{\text{ECP}_{\text{IL-5}}}$$

where $\text{ECP}_{\text{IL-5}}$ = ng/ml of rhIL-5-enhanced ECP release in the presence of drug vehicle, and $\text{ECP}_{\text{IL-5+drug}}$ = ng/ml of rhIL-5-enhanced ECP release in the presence of drug.

Statistical differences between groups of data were determined by Student's *t*-test and paired *t*-test (Fig. 5) with the aid of a statistical package (Minitab Release 7, Minitab Inc., State College, PA, USA). The effects of rapamycin, cyclosporin A, and dexamethasone (Figs. 2 and 6) were analysed by one-way analysis of variance, followed by paired comparisons. *P* values <0.05 were considered significant.

Results

Rapamycin, cyclosporin A, and dexamethasone suppressed rhIL-5-enhanced survival of eosinophils

The effects of rapamycin, cyclosporin A, and dexamethasone on rhIL-5-enhanced eosinophil survival are shown in Fig. 1. After 4 days, only 2% of MACS purified human peripheral blood eosinophils were viable when cultured with medium control (Fig. 1). In the presence of 10 ng/ml rhIL-5, $72.0 \pm 3.2\%$ (mean $\pm$ SEM) of eosinophils survived for 3 days, and enhanced survival was maintained for up to 5 days. IL-5-enhanced eosinophil survival was inhibited in the presence of

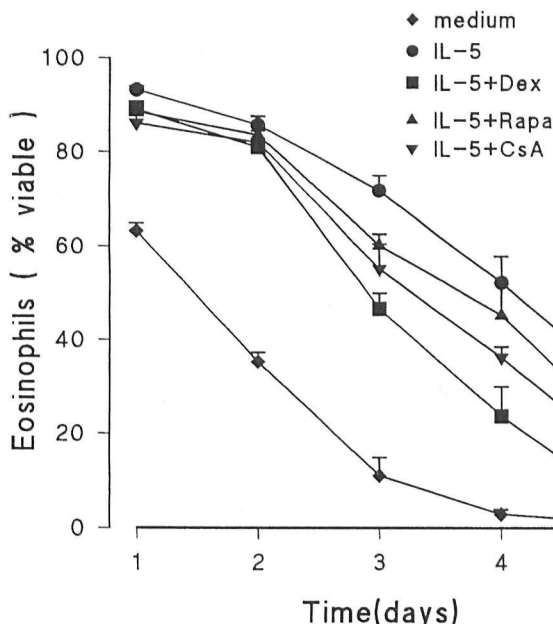


Fig. 1. Time course of effects of rapamycin (Rapa), cyclosporin A (CsA), and dexamethasone (Dex) (all at 10^{-6} M) on rhIL-5 (10 ng/ml)-enhanced eosinophil ($2 \times 10^6/\text{ml}$) survival. Viability of eosinophils was analysed by flow cytometry with propidium iodide exclusion. Results were expressed as percentages (mean $\pm$ SEM) of viable eosinophils ($n=4$).

rapamycin, cyclosporin A, and dexamethasone (all at 10^{-6} M). Inhibition was statistically significant after 72 h of culture, but not earlier. In the absence of rhIL-5, none of the three compounds (at concentrations of 10^{-9} – 10^{-6} M, 24-h culture) significantly influenced eosinophil viability (data not shown). At 72 h, rapamycin and dexamethasone exhibited concentration-dependent inhibition of rhIL-5-enhanced eosinophil survival (Fig. 2) (one-way analysis of variance, Rapa: $P=0.017$; Dex: $P=0.001$). Cyclosporin A also showed some inhibition of rhIL-5-enhanced survival of eosinophils, although the concentration-response effect was not statistically significant.

Rapamycin, cyclosporin A, and dexamethasone inhibited rhIL-5-enhanced ECP release

The effects of the drugs on eosinophil degranulation were evaluated by measurement of ECP release. Exposure of eosinophils to serum-, but not control HBSS-treated Sephadex beads resulted in a time-dependent release of ECP in the absence of rhIL-5 (Fig. 3). When eosinophils were preincubated (45 min, 20 ng/ml) with rhIL-5 and then stimulated (30 min) with serum-treated beads, the release of ECP (absolute concentrations in supernatants and percent release of total ECP) was significantly enhanced (Fig. 4) (paired t -test, $P=0.0024$ and $P=0.0008$, respectively). When eosi-

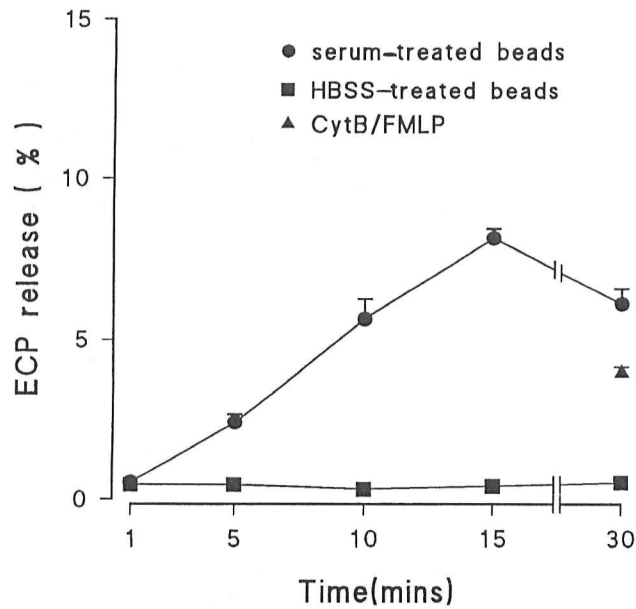


Fig. 3. Time course of ECP release from purified human blood eosinophils after stimulation with serum-treated beads. Eosinophils were incubated with cytochalasin B (CytB, 5 lg/ml) and FMLP (10^{-6} M) for 30 min under same conditions as positive control. HBSS-treated beads were used as negative control. Released ECP was expressed as percentage of total released by freeze-thawed cells (mean $\pm$ SEM, $n=6$).

nophils were preincubated (45 min) with medium control, all three drugs (10^{-6} – 10^{-9} M) did not significantly inhibit ECP release when stimulated (30 min) with serum-treated beads (data not shown). However, ECP release by eosinophils preincubated (45 min) with rhIL-5 was significantly inhibited by rapamycin and cyclosporin A in a concentration-dependent fashion (Fig. 5) (one-way analysis of variance, Rapa: $P=0.031$; CsA=0.035). Dexamethasone

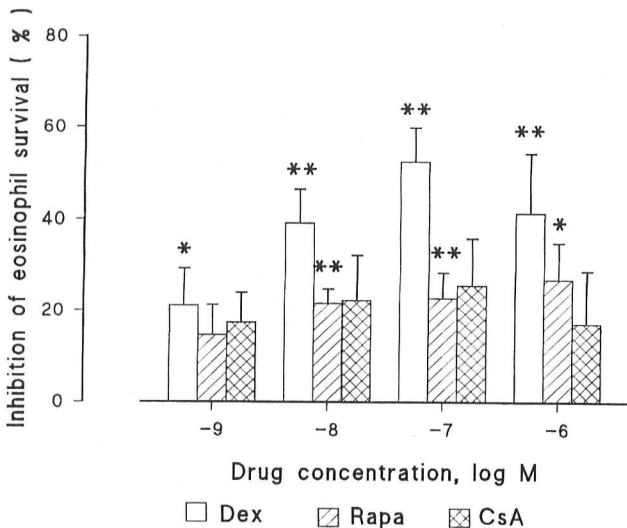


Fig. 2. Dose-response relationships (mean $\pm$ SEM) of effects of rapamycin, cyclosporin A, and dexamethasone on rhIL-5-enhanced eosinophil survival (time=72 h, $n=6$). Drug-induced inhibition of IL-5-enhanced survival was determined by formula: inhibition of survival (%) = $(V_{IL-5} - V_{IL-5+drug}) / V_{IL-5} \times 100\%$, where V_{IL-5} = % viability in presence of rhIL-5 (10 ng/ml) and drug vehicle, and $V_{IL-5+drug}$ = % viability in presence of rhIL-5 and drug. * $P<0.05$, ** $P<0.01$ (vs drug vehicle control).

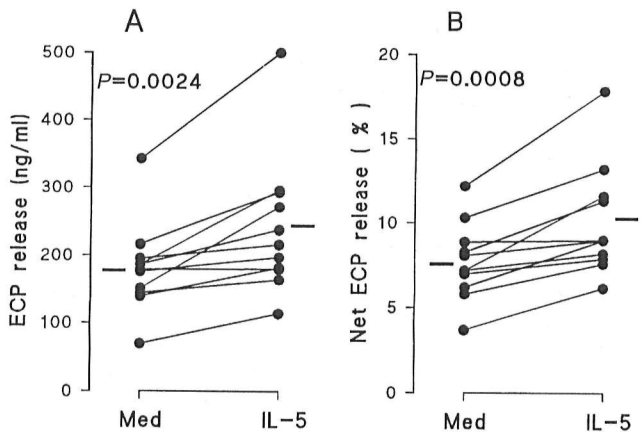


Fig. 4. Recombinant human IL-5-enhanced ECP release from eosinophils. Eosinophils were preincubated (45 min, 37°C) with rhIL-5 (20 ng/ml) or medium control, and then stimulated (30 min) with serum-treated beads. ECP release was expressed as absolute supernatant concentrations (ng/ml) or percentages of total release from freeze-thawed cells (mean $\pm$ SEM, $n=11$).

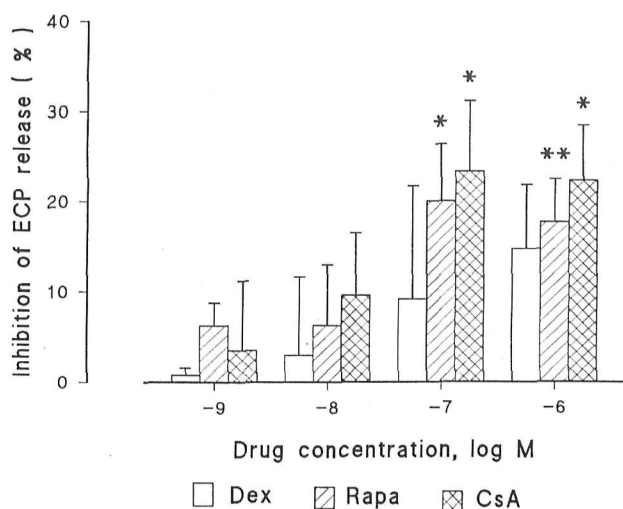


Fig. 5. Concentration-dependent inhibition of rhIL-5-enhanced eosinophil ECP release by rapamycin, cyclosporin A, and dexamethasone (mean $\pm$ SEM; $n=7$). Eosinophils were pre-incubated (45 min, 37°C) with rhIL-5 (20 ng/ml) and serial dilutions of drugs or vehicle control, and then stimulated (30 min) with serum-treated beads. Inhibition was determined by formula: inhibition of release (%) = $(ECP_{IL-5} - ECP_{IL-5+drug}) / ECP_{IL-5} \times 100\%$, where ECP_{IL-5} = ng/ml of rhIL-5-enhanced ECP release in presence of drug vehicle, and $ECP_{IL-5+drug}$ = ng/ml of rhIL-5-enhanced ECP release in presence of drug. * $P < 0.05$; ** $P < 0.01$ (vs drug vehicle control).

also showed some inhibition, but the concentration-response effect was not statistically significant. At the concentration of 10^{-7} M, cyclosporin A and rapamycin inhibited rhIL-5-induced ECP release by 23.4% and 20.1%, respectively (Fig. 5, $P < 0.05$).

Discussion

Dexamethasone and rapamycin showed concentration-dependent inhibition of IL-5-enhanced eosinophil survival, whereas cyclosporin A did not (Figs. 1 and 2). Dexamethasone and rapamycin showed no effect on eosinophil survival, over a range of pharmacologic concentrations, in the absence of IL-5. This suggests a direct inhibitory effect of these drugs on IL-5-mediated signalling events in eosinophils resulting in prolonged survival, although the possibility of additional effects (such as inhibition of endogenous synthesis of survival-prolonging cytokines by eosinophils [16]) cannot be excluded. The fact that the effects of these drugs varied between subjects suggests that at least some eosinophils in some subjects may have been activated by cytokines such as IL-5 *in vivo* (it has been suggested, for example, that "hypodense" eosinophils, which form a proportion of the total circulating eosinophil population in atopic and asthmatic subjects, may have been activated in this way [19–21]). This might have par-

tially abrogated some of the effect of exogenous IL-5 observed on eosinophils obtained *ex vivo* and consequently the ability of these drugs to inhibit these effects. On the other hand, the failure of the drugs to exert significant inhibitory activity in the absence of exogenous IL-5 argues against this possibility. Such considerations do not, however, detract from our novel observation that rapamycin inhibits IL-5-enhanced eosinophil survival.

Our data confirm and extend previous reports (22, 23) of an inhibitory effect of dexamethasone on the survival of eosinophils enhanced by GM-CSF and IL-5. Although these studies showed some inhibitory effect of dexamethasone on eosinophil survival in the absence of exogenous cytokines, this was only at very high concentrations ($>10^{-5}$ M), arguably reflecting nonspecific toxic, rather than pharmacologic effects.

Our demonstration that cyclosporin A failed to inhibit IL-5-enhanced survival of eosinophils *in vitro* is novel, although a previous study showed that the immunosuppressive drug FK506 was ineffective in this regard (24). Although cyclosporin A and FK506 bind to distinct intracellular immunophilin receptors, their range of inhibitory activities, at least on T cells, have been shown to be remarkably similar (25, 26), and this would appear to be reflected in the lack of inhibition by both drugs of IL-5-enhanced eosinophil survival.

In addition, we have shown that prior short-term exposure of eosinophils to IL-5 *in vitro* results in significant enhancement of eosinophil degranulation, as measured by ECP release, on exposure to "opsonized" Sephadex beads. These beads presumably activate eosinophils at least partly by engagement and cross-linking of surface IgG, IgA, and IgE Fc receptors, a process which may be relevant to eosinophil degranulation within the mucosal surface *in vivo*, although this hypothesis remains unexplored. Similarly, "priming" of eosinophils with IL-5 *in vitro* resulted in elevated phorbol ester-induced superoxide generation (27) and immunoglobulin-induced release of the granule protein EDN (28). Both cyclosporin A and rapamycin significantly inhibited this effect in a concentration-dependent fashion at a range of pharmacologic concentrations, whereas dexamethasone did not. Again, the fact that these effects were observed in the presence, but not the absence, of IL-5 suggests a direct inhibitory effect of these drugs on IL-5-mediated signalling events in eosinophils resulting in elevated degranulation, and the fact that these effects were somewhat variable suggests the possibility of a degree of eosinophil priming *in vivo* in some subjects. Our observed lack of effect of dexamethasone in this regard is consistent with a previous study (28).

Little is known of the mechanisms whereby IL-5 enhances eosinophil degranulation, although there is some evidence that this involves rapid modulation of second messengers such as protein kinase C without the necessity of new protein synthesis (29).

The differential effects of dexamethasone, cyclosporin A, and rapamycin on IL-5-enhanced eosinophil viability on the one hand and degranulation on the other are consistent with the hypothesis that these drugs interfere with IL-5-mediated signalling events in eosinophils by distinct mechanisms, although in view of the paucity of knowledge of the nature of these events, any further interpretation of these facts would seem premature at present.

However, our data do clearly demonstrate inhibitory effects of these drugs at pharmacologically relevant concentrations (30) on IL-5-mediated selective eosinophil survival and enhanced release of toxic granule proteins, two processes which are regarded as key events in the pathogenesis of allergic and asthmatic inflammation. Although these effects are small in terms of percent inhibition, they might contribute to amelioration of asthma, particularly if employed for chronic therapy. These observations, taken together with the direct inhibitory effects of these drugs on activated T cells (30), which are considered a major source of local IL-5 in these diseases (31), would seem to justify continued appraisal of these drugs as antiallergy and antiasthma agents (10).

Acknowledgments

This work was supported by the Medical Research Council (UK) and Wyeth-Ayerst (USA).

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