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Cyclic AMP-elevating agents prolong or inhibit eosinophil survival depending on prior exposure to GM-CSF

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1 Purified human eosinophils survived for up to 7 days when cultured *in vitro* in the presence of 1 ng ml^{-1} granulocyte-macrophage colony stimulating factor (GM-CSF) with a viability of 73%. In the absence of GM-CSF, eosinophil viability decreased after one day in culture, and only 4% of cells were viable by day 4.

2 Culture of eosinophils with cholera toxin produced a concentration-dependent decrease in GM-CSF-induced survival at 7 days (IC_{50} = 7 ng ml^{-1}) which was associated with a 6 fold increase in the intracellular cyclic AMP concentration. This inhibition of cell survival could be prevented by the addition of the protein kinase A inhibitor, H89 (10^{-6}M).

3 When eosinophils were cultured with dibutyryl cyclic AMP, there was a concentration-dependent inhibition of GM-CSF-induced survival at 7 days with an IC_{50} of 200 μM. The related cyclic nucleotide analogue, dibutyryl cyclic GMP did not inhibit GM-CSF-induced eosinophil survival over the same concentration range.

4 Culture of eosinophils with forskolin, or with the phosphodiesterase inhibitors, rolipram and SK&F94120, had no effect on GM-CSF-induced eosinophil survival at any concentration examined.

5 After 7 days' culture in the absence of GM-CSF, fractionation of eosinophil DNA on agarose gels demonstrated a 'ladder' pattern characteristic of apoptosis. GM-CSF prevented DNA fragmentation and this protection could be overcome by both cholera toxin and dibutyryl cyclic AMP.

6 GM-CSF did not affect intracellular cyclic AMP concentrations in unstimulated eosinophils or in cells stimulated by cholera toxin. Thus, GM-CSF does not apparently increase eosinophil survival by affecting cyclic AMP levels.

7 In the absence of GM-CSF both cholera toxin and dibutyryl cyclic AMP decreased the rate of eosinophil death, when compared to cells cutured with medium alone. The $t_{1/2}$ values for cell death were 1.63 ± 0.3, 2.46 ± 0.3 and 4.62 ± 1.0 days for cells cultured in the presence of medium, cholera toxin and dibutyryl cyclic AMP respectively.

8 In conclusion, cyclic AMP exerts opposing effects on eosinophil survival depending on prior exposure of the cells to GM-CSF.

Keywords: Eosinophil; GM-CSF; cell survival; signal transduction; cyclic AMP; apoptosis; cytokine

Introduction

Bronchial asthma is characterized by increased numbers of eosinophils in the peripheral blood, bronchial tissues and sputum (Dunhill, 1960; Lowell, 1967; Franklin, 1974; Horn *et al.*, 1975). The extent of the peripheral blood eosinophilia correlates with the degree of airway hyperreactivity (Durham & Kay, 1985; Taylor & Luksza, 1987) and the severity of the disease (Bousquet *et al.*, 1990). Eosinophils release preformed and newly synthesized mediators, including eosinophil cationic protein, major basic protein and leukotriene C_4, that may induce many of the airway changes characteristic of the pathology of asthma. For these reasons eosinophils are believed to be the key effector cell mediating damage to airway epithelial cells and nerves (Frigas & Gleich, 1986).

Recent evidence has established that stimulation of eosinophil differentiation from precursor cells, recruitment of cells into tissues and increased survival and activation of eosinophils are modulated by the influence of various haemopoetic cytokines, which include granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3) and interleukin-5 (IL-5). We have demonstrated previously that GM-CSF represents a major eosinophil activating activity produced by both alveolar macrophages (Howell *et al.*, 1989) and peripheral blood mononuclear cells (Burke *et al.*, 1991) obtained from asthmatic patients. Increased levels of GM-CSF have been detected in both the bronchoalveolar lavage fluid (Mattoli *et al.*, 1991) and airway epithelial cells (Sousa *et al.*, 1993) of asthmatic individuals. Similarly, increased numbers of bronchoalveolar lavage cells expressing mRNA for GM-CSF, IL-3 and IL-5 have been observed in subjects with symptomatic asthma (Marini *et al.*, 1992; Robinson *et al.*, 1993). Collectively, these results suggest that there is upregulation of cytokine expression in bronchial asthma, which may in turn influence eosinophil survival and function.

Recent reports have shown that eosinophils (Tai *et al.*, 1991; Stern *et al.*, 1992) and haemopoetic precursor cells (Williams *et al.*, 1990) maintained in culture without the addition of exogenous cytokines undergo a 'programmed cell death'. This has the morphological characteristics of apoptosis and DNA is cleaved into oligonucleosome length fragments, giving a characteristic 'ladder' pattern when visualized on agarose gels. Apoptotic cells are recognised and ingested by phagocytic macrophages (Stern *et al.*, 1992). This action would be anticipated to facilitate the removal of eosinophils from the tissue by phagocytes, without the release of the toxic granule components; a mechanism which may be of significance in the

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control of inflammation. The addition of GM-CSF, IL-5 or IL-3 to cultures of eosinophils prevents apoptotic death (Williams *et al.*, 1990; Tai *et al.*, 1991; Stern *et al.*, 1992). The second messenger events which control apoptosis remain unclear although several agents have been shown to induce apoptosis in various cell types. One well characterized phenomenon is the ability of corticosteroids to bring about apoptotic death in thymocytes (Wyllie, 1980). Further, agents that elevate the intracellular level of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) also induce apoptosis in cells such as thymocytes (MacConkey *et al.*, 1990) and certain myeloid cell lines, including IPC-81 (Lanotte *et al.*, 1991).

Previous studies have established that the second messenger cyclic AMP can regulate certain eosinophilic functions, such as the inhibition of degranulation (Kita *et al.*, 1991), thromboxane generation (Souness *et al.*, 1994), leukotriene C_4 generation (Munoz *et al.*, 1994) and superoxide anion production (Dent *et al.*, 1991; 1994). In this study we have assessed whether cyclic AMP promotes apoptotic cell death in human eosinophils cultured with GM-CSF.

Methods

Eosinophil purification and culture

Eosinophils were isolated from 100 ml EDTA (0.2 M)-anticoagulated peripheral blood from patients who had bronchial asthma, allergic rhinitis or parasite infection. After sedimentation of the red cells with 0.2 vol. 6% dextran (mol. wt. 162,000) in 0.9% saline for 45 min at room temperature, the leukocyte-rich plasma was aspirated and eosinophils were separated by depletion of neutrophils with CD16 coated magnetic microbeads using the MACS isolation system according to the method of Hansel *et al.* (1991). Briefly, the leukocytes were washed in Minimal Essential Medium (MEM) + 2% foetal bovine serum (FBS) by centrifugation at 400 *g* for 10 min and were resuspended in 10 ml MEM/FBS. The cells were layered on to 20 ml of Percoll (density 1.088 g ml^{-1}) and centrifuged at 1000 *g* for 30 min. The mononuclear cell layer was carefully removed along with the remaining Percoll and the granulocyte pellet was resuspended in 1 ml MEM/FBS and counted in Kimura stain. The CD16 microbeads were added to the cell suspension at a ratio of 50 μl per 5×10^7 neutrophils and the cells were incubated for 45 min on ice. The cell suspension was then added to the top of the MACS separation column in a magnetic field and the eosinophils were washed through with 30 ml MEM/FBS. The column eluate was centrifuged at 400 *g* for 10 min, the cell pellet was resuspended in 1 ml MEM/FBS and was counted in Kimura stain. Eosinophils of greater than 98% purity were used in all experiments.

Freshly isolated eosinophils were resuspended at a concentration of 2×10^6 cells ml^{-1} in RPMI 1640 supplemented with 25 mM HEPES, 32 mM L-glutamine, penicillin-streptomycin (100 u ml^{-1} and 100 μg ml^{-1}) and 10% FBS (supplemented RPMI). Twenty-five microlitres of the cell suspension were cultured in a 96-well plastic tissue culture plate containing 75 μl supplemented RPMI with 1 ng ml^{-1} recombinant human GM-CSF and various concentrations of the agents under investigation, added simultaneously with the GM-CSF. The cells were cultured for a period of 7 days after which viability was assessed in duplicate wells by trypan blue exclusion. In each experiment, eosinophils were cultured in the presence of 1 ng ml^{-1} GM-CSF alone as a positive control and supplemented RPMI alone as a negative control.

Cyclic AMP measurements

One million purified eosinophils were resuspended in 300 μl supplemented RPMI containing the agents under investigation in a 24-well plastic tissue culture plate and were incubated at 37°C, 5% CO_2 for various periods of time. The incubation was stopped by the addition of 300 μl 1 M trichloroacetic acid to each well and the plate was incubated on ice for approximately 20 min. The contents of each well were transferred to 1.5 ml plastic centrifuge tubes and cyclic nucleotides were extracted according to the method of Downes *et al.* (1986). Briefly, the tubes were vortex-mixed, centrifuged at 10000 *g* for 5 min and 500 μl of supernatant was removed and added to a clean tube containing 50 μl 25 mM EDTA. The contents of the tube were vortex-mixed and 500 μl of a 1:1 mixture of 1,1,2-trichlorotrifluoroethane:trioctylamine was added. The tubes were vortex-mixed again (at least 15 s per tube) and centrifuged at 10000 *g* for 3 min; 450 μl of the upper, aqueous phase was carefully removed and placed in a clean tube containing 50 μl 120 mM $NaHCO_3$ and the samples were stored at −70°C. Cyclic AMP levels were measured, after acetylation, in duplicate samples with a commercially available Enzyme Immunoassay kit, following the manufacturer's instructions.

DNA extraction and electrophoresis

DNA was extracted according to the method of Miller *et al.* (1988). Approximately 4×10^6 eosinophils were harvested from culture and washed in supplemented RPMI (without FBS), the red cells were then lysed with ammonium chloride lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4) for 10 min on ice. The eosinophils were washed in supplemented RPMI (without FBS) by centrifugation at 400 *g* for 10 min and were resuspended in 15 ml polypropylene centrifugation tubes with 3 ml nuclei lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na_2EDTA, pH 8.2), 0.2 ml 10% sodium dodecyl sulphate (SDS) 2 and 0.5 ml proteinase K solution (1 mg proteinase K in 1% SDS and 2 mM Na_2EDTA) and were incubated at 37°C overnight. Protein was precipitated by adding 1 ml saturated NaCl (approx. 6 M) and shaking the tube vigorously for 15 s. The protein was removed by centrifugation at 3000 *g* for 15 min. The DNA was precipitated by adding two volumes of absolute ethanol at 20°C and mixing by inversion. The tubes were centrifuged at 13000 *g* and the precipitates were washed in 70% ethanol and air dried at room temperature. The DNA was then allowed to dissolve in 50 μl TE buffer (20 mM Tris-HCl, 5 mM EDTA, pH 7.6) overnight at 4°C. Two microlitres of loading buffer (20 mM, Tris-HCl, 5 mM EDTA, 50% glycerol, 0.05% Orange G) was added to 1 μg DNA and the samples were heated at 65°C for 5 min. The samples were loaded onto a 1.8% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide (1 μg ml^{-1}) and were electrophoresed at 50 V for 2 h before viewing under u.v. light.

Materials

Cholera toxin, dibutyryl cyclic AMP, dibutyryl cyclic GMP, dextran and trichloroacetic acid were obtained from Sigma Chemical Co. (Poole, U.K.). CD16 microbeads and the MACS isolation system were obtained from Eurogenetics Ltd. (Teddington, U.K.). All culture media and additives, and tissue culture plastic-ware were obtained from Life Technologies Ltd. (Paisley, U.K.). Foetal bovine serum was obtained from Globepharm Ltd. (Esher, U.K.). Percoll was obtained from Pharmacia (Milton Keynes, U.K.). Recombinant human GM-CSF was obtained from Boehringer Mannheim (Lewes, U.K.). Trioctylamine and 1,1,2-trichlorotrifluoroethane were obtained from Aldrich Chemical Co. (Gillingham, U.K.). Cyclic AMP immunoassay kits were obtained from Amersham International plc (Little Chalfont, U.K.). Forskolin and N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulphonamide (H89) were obtained from Calbiochem (Nottingham, U.K.). Rolipram was a gift from Dr H. Wachtal, Schering AG (Berlin, Germany) and 5-(4-acetamidophyl pyrazin-2(1H)-one (SKF94120) was a gift from Dr B. Warrington, Smith Kline Beecham (Welwyn, U.K.). All other chemicals were obtained from BDH Laboratory Supplies (Poole, U.K.).

Results

Effect of GM-CSF on eosinophil survival

Purified eosinophils survived for up to 7 days when cultured in the presence of 1 ng ml^{-1} GM-CSF, with a viability of $73 \pm 4\%$ on day seven. In the absence of GM-CSF, however, eosinophil survival began to decrease after 1 day in culture, with a viability of $45 \pm 1\%$ on day two, dropping to $4 \pm 1\%$ by day four ($n = 3$).

Effect of cholera toxin on eosinophil survival

Culture of eosinophils in the presence of GM-CSF (1 ng ml^{-1}) and cholera toxin (1 pg ml^{-1} to 1 μg ml^{-1}) resulted in a concentration-dependent decrease in survival at 7 days, with a threshold at 100 pg ml^{-1} cholera toxin and an IC_{50} of 7 ng ml^{-1} (Figure 1). Eosinophil survival had decreased maximally to $39 \pm 10\%$ viability at 10 ng ml^{-1} cholera toxin. Increasing the concentration of cholera toxin to 1 μg ml^{-1} did not decrease eosinophil survival further.

In order to verify that cholera toxin increased cyclic AMP in human eosinophils, intracellular cyclic AMP levels were measured in cells which had been in culture for 24 h (Figure 2). At this time point, eosinophils were still viable, even in the absence of GM-CSF. In the absence of GM-CSF or cholera toxin, the intracellular cyclic AMP concentration was 61 ± 2 fmol/10^6 cells and in the presence of GM-CSF the intracellular cyclic AMP level was 59 ± 12 fmol/10^6 cells. Exposure of eosinophils to cholera toxin (1 pg ml^{-1} – 1 μg ml^{-1}) evoked a concentration-dependent increase in the cyclic AMP content, with maximal effect at 1 μg ml^{-1} (455 ± 50 fmol/10^6 cells in the absence of GM-CSF; 515 ± 98 fmol/10^6 cells in the presence of GM-CSF). There was no difference in the cholera toxin concentration-response curves between eosinophils cultured in the absence or presence of GM-CSF.

Addition of the protein kinase A (PKA) inhibitor, H89, to eosinophils cultured with GM-CSF and cholera toxin (10 ng ml^{-1}) attenuated the inhibitory effect of cholera toxin on eosinophil survival in a concentration-dependent manner, with maximal effect at 10 $^{-6}$M H89 (Table 1). H89 alone had no effect on eosinophil survival at concentrations up to 10 $^{-6}$M.

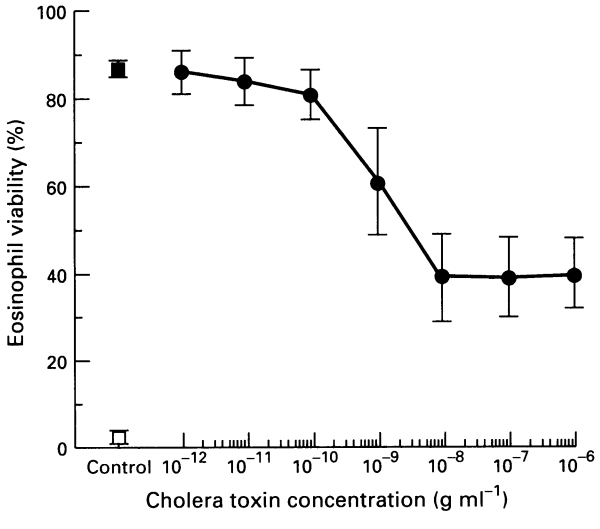

Figure 1 The effect of cholera toxin (●) on GM-CSF-induced eosinophil survival at 7 days. The positive control (■) is incubation with 1 ng ml^{-1} GM-CSF and the negative control (□) is incubation with culture medium alone. Each point is the mean ± s.e.mean of 4 experiments.

Effect of cyclic nucleotide analogues on eosinophil survival

Culture of eosinophils with dibutyryl cyclic AMP (10^{-7}–10^{-3} M), a stable, non-hydrolysable analogue of cyclic AMP, inhibited eosinophil survival in a concentration-dependent manner, with an IC_{50} of 200 μM (Figure 3). In contrast, the related cyclic nucleotide analogue, dibutyryl cyclic GMP, which was included as a control in each experiment, failed to inhibit eosinophil survival over the same concentration range.

Effect of forskolin on eosinophil survival

Addition of the adenylyl cyclase activator, forskolin, to eosinophils cultured in the presence of 1 ng ml^{-1} GM-CSF over 7 days had no effect on survival over the concentration range (10^{-9}–10^{-5}M) studied. Similarly, the cyclic AMP content of eosinophils cultured for 24 h with GM-CSF was unaffected by forskolin, except at the highest concentration (10^{-5}M) examined, where a modest increase from 69 ± 20 to 181 ± 30 fmol/10^6 cells, was observed ($n = 5$).

Effect of phosphodiesterase inhibitors on eosinophil survival

The predominant, if not exclusive, cyclic AMP hydrolytic activity in human eosinophils is a member of the phosphodiesterase (PDE) 4 isoenzyme family (Dent *et al.*, 1994; Hatzelmann *et al.*, 1995). We therefore assessed the effect of

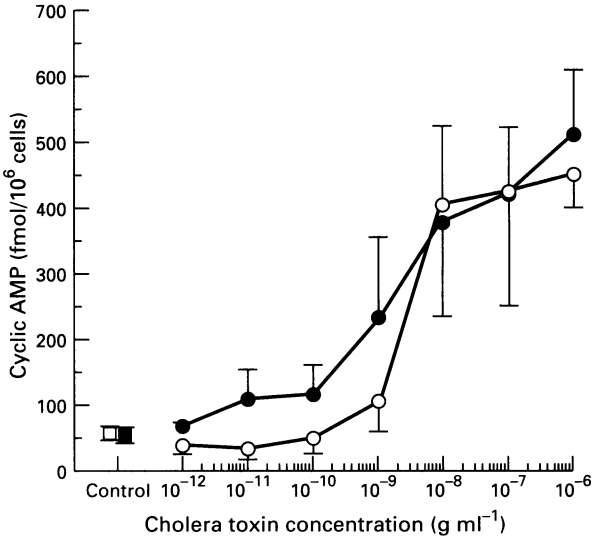

Figure 2 The effect of cholera toxin on eosinophil cyclic AMP levels cultured in the absence (○) and presence (●) of 1 ng ml^{-1} GM-CSF, measured at 24 h. In the absence of cholera toxin cyclic AMP levels were 61 ± 2 fmol/10^6 cells and 59 ± 12 fmol/10^6 cells in the absence (□) and presence (■) of GM-CSF respectively. Each point is the mean ± s.e.mean of 3 experiments.

Table 1 Effect of cholera toxin and H89 on GM-CSF-induced eosinophil survival after 7 days in culture

	Viability (%)
Medium control	2 ± 1
GM-CSF (1 ng ml^{-1})	79 ± 2
GM-CSF + cholera toxin (10 ng ml^{-1})	47 ± 3
GM-CSF + cholera toxin + H89 (10^{-6} M)	71 ± 4

Values are mean ± s.e.mean; $n = 4$.

the PDE4 inhibitor, rolipram and, as a control, the PDE3 inhibitor, SK&F94120 on eosinophil survival. Neither rolipram nor SK&F94120 had any effect on GM-CSF-induced eosinophil survival at 7 days, over the concentration range 10^{-10}–10^{-6}M ($n=4$). Cyclic AMP was measured at 24 h in cultures with GM-CSF and rolipram over the same concentration range and we found that rolipram induced only a small increase in intracellular cyclic AMP levels from a control of 80 ± 26 fmol/10^6 cells with GM-CSF alone, reaching a maximum of 121 ± 38 fmol/10^6 cells at 10^{-6}M rolipram ($n=3$).

Time course of cyclic AMP levels

Cyclic AMP levels were measured over the first 24 h of culture, a point at which the cells were >98% viable even in the absence of GM-CSF (Figure 4). There was no change in cyclic AMP levels in unstimulated or GM-CSF-stimulated eosinophils. Cholera toxin (10 ng ml^{-1}) induced a time-dependent increase in cyclic AMP levels, whereas forskolin (10^{-5}M) did not affect cyclic AMP levels compared to those observed with either unstimulated or GM-CSF stimulated cells.

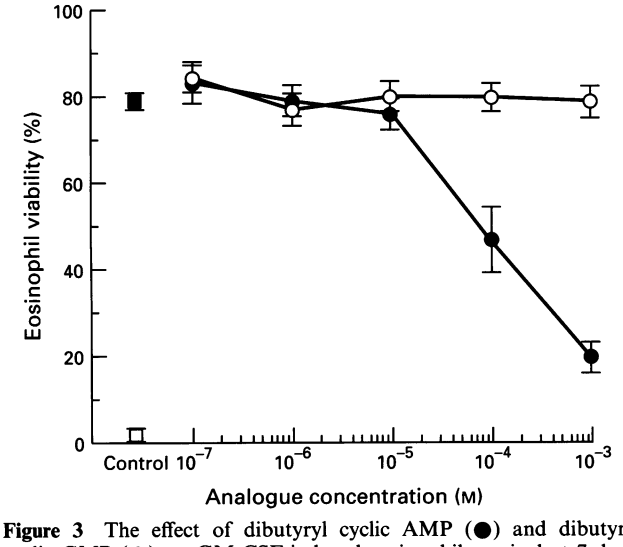

Figure 3 The effect of dibutyryl cyclic AMP (●) and dibutyryl cyclic GMP (○) on GM-CSF-induced eosinophil survival at 7 days. The positive control (■) is incubation with 1 ng ml^{-1} GM-CSF and the negative control (□) is incubation with culture medium alone. Each point is the mean±s.e.mean of 3 experiments.

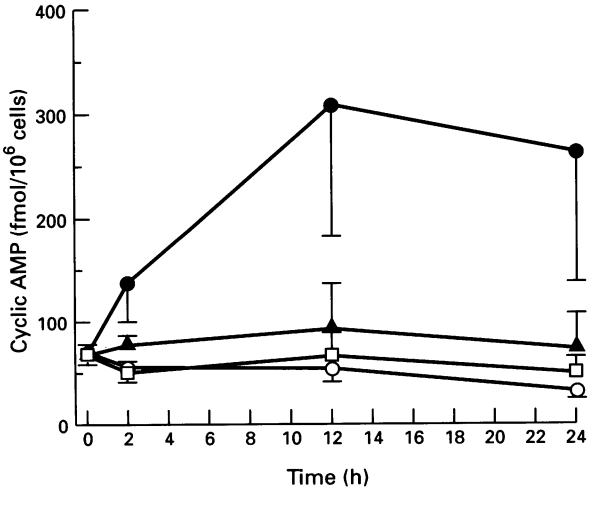

Figure 4 Time course of cyclic AMP levels of eosinophils cultured in medium alone (○), 1 ng ml^{-1} GM-CSF (□), GM-CSF + 10 ng ml^{-1} cholera toxin (●) and GM-CSF + 10^{-5}M forskolin (▲). Each point is the mean±s.e.mean of 3 experiments.

Effect of GM-CSF, cholera toxin and dibutyryl cyclic AMP on DNA fragmentation

After 1 day culture in the absence of GM-CSF, eosinophil viability began to decrease and this was accompanied by fragmentation of DNA (data not shown). When eosinophils were cultured with GM-CSF both cholera toxin (10 ng ml^{-1}) and dibutyryl cyclic AMP (10^{-3}M) inhibited eosinophil survival in a time-dependent manner, with the inhibition beginning after 4 days ($n=5$, data not shown). We therefore examined DNA from eosinophils cultured for 7 days (Figure 5), which was the time when viability was routinely assessed, and was associated with a 50–70% reduction in survival. In the absence of GM-CSF, eosinophil DNA run on an agarose gel was completely fragmented, showing a distinctive 'ladder pattern' of approximately 200 base pair fragments indicating the activation of an endonuclease activity characteristic of apoptosis. GM-CSF (1 ng ml^{-1}) prevented this DNA breakdown, however the presence of dibutyryl cyclic AMP (10^{-3}M) or cholera toxin (10 ng ml^{-1}) was able to overcome the effects of GM-CSF. Forskolin did not induce any breakdown of DNA.

Effects of GM-CSF on cyclic AMP levels

To test further the hypothesis that GM-CSF may increase eosinophil survival by decreasing intracellular cyclic AMP concentrations, cyclic AMP levels induced by 1 ng ml^{-1} cholera toxin, a concentration that produced sub-maximal decreases in cell viability, were measured at 24 h in the presence of increasing concentrations of GM-CSF (Figure 6). Cholera toxin (1 ng ml^{-1}) increased cyclic AMP levels from a control of 61 ± 1 fmol/10^6 cells, when cells were cultured in medium alone, to 179 ± 56 fmol/10^6 cells and increasing concentrations of GM-CSF did not reduce the levels of cyclic AMP stimulated by cholera toxin.

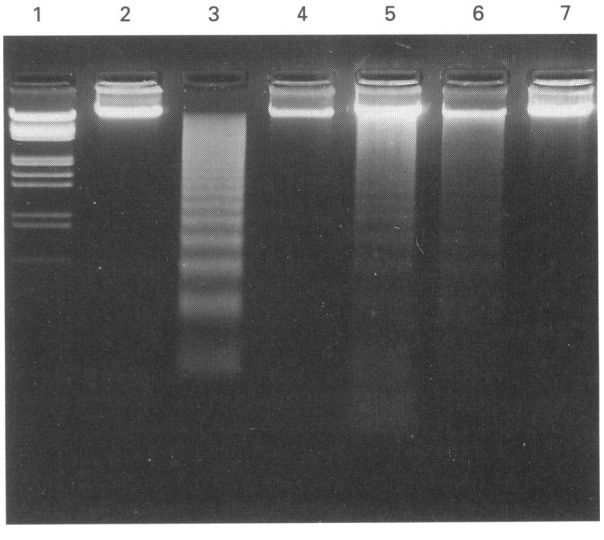

Figure 5 Agarose gel electrophoresis of DNA extracted from human eosinophils. *Lane 1* contains *Hin* d III + *Eco* RI DNA markers. *Lane 2* contains DNA from freshly isolated eosinophils. *Lanes 3–7* contain DNA extracted from eosinophils cultured for 7 days with: supplemented RPMI alone (*lane 3*); 1 ng ml^{-1} GM-CSF alone (*lane 4*); GM-CSF and dibutyryl cyclic AMP (10^{-3}M) (*lane 5*); GM-CSF and cholera toxin (10 ng ml^{-1}) (*lane 6*); GM-CSF and forksolin (10^{-5}M) (*lane 7*).

Time course of eosinophil survival with cholera toxin and dibutyryl cyclic AMP in the absence of GM-CSF

In order to investigate whether the effects of GM-CSF and cyclic AMP-elevating agents represent two quite separate processes we assessed the effects of cholera toxin and dibutyryl cyclic AMP on eosinophil survival in the absence of GM-CSF (Figure 7). We found that both cholera toxin (10 ng ml^{-1}) and dibutyryl cyclic AMP (10^{-3}M) decreased the rate of eosinophil death when compared to cells cultured in medium alone. Thus, the times taken for 50% of the eosinophils to die ($t_{1/2}$) were 1.63 ± 0.3 days for cells cultured in medium alone, 2.46 ± 0.3 days for cells cultured with cholera toxin and 4.62 ± 1.0 days for cells cultured with dibutyryl cyclic AMP. The control compound, dibutyryl cyclic GMP, had no effect on eosinophil survival in the absence of GM-CSF ($n=3$, data not shown). Addition of the PKA inhibitor, H89 (10^{-6}M) to the cultures had no effect on eosinophil survival alone, ($n=3$, data not shown) or on the changes induced by either cholera toxin (10 ng ml^{-1}) (Figure 8a) or dibutyryl cyclic AMP (10^{-3}M) (Figure 8b). To determine whether the eosinophil survival induced by cholera toxin and dibutyryl cyclic AMP, in the absence of GM-CSF, was limited by the stability of the compounds, cells were cultured for 6 days, with renewal of the medium, GM-CSF and cyclic AMP-elevating agents each day. No differences were observed in the rate of eosinophil death between these cells and the same cells cultured without any change of medium ($n=3$, data not shown).

Discussion

There is emerging evidence of the importance of 'programmed cell death' and apoptosis in the control of cellular turnover and the regulation of disease. The evidence that increases in the intracellular levels of cyclic AMP have been associated with the induction of apoptosis and the reduction of survival in certain cell types such as thymocytes (MacConkey *et al.*, 1990) and a myeloid cell line IPC-81 (Lanotte *et al.*, 1991) prompted an assessment of the effects of cyclic AMP on eosinophil survival. We have shown that the survival of human eosinophils can be decreased by adding cholera toxin or dibutyryl cyclic AMP to cells cultured with GM-CSF. Both of these compounds increase intracellular cyclic AMP levels, through independent mechanisms. Cholera toxin increases cyclic AMP by interacting with G_s, whereas dibutyryl cyclic AMP, a stable, non-hy-

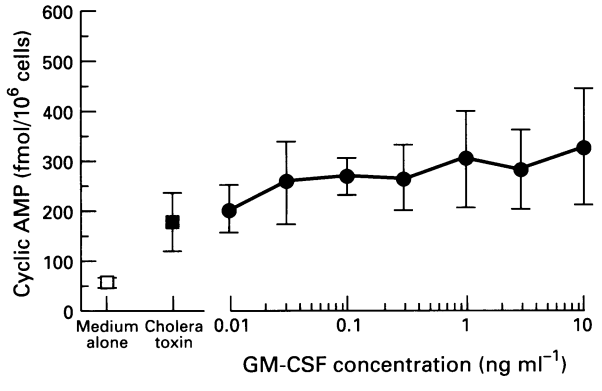

Figure 6 The effect of GM-CSF on cyclic AMP levels induced by 1 ng ml^{-1} cholera toxin at 24 h. The levels of cyclic AMP measured with culture medium alone and with 1 ng ml^{-1} cholera toxin are also shown. Each point is the mean ± s.e.mean of 4 experiments.

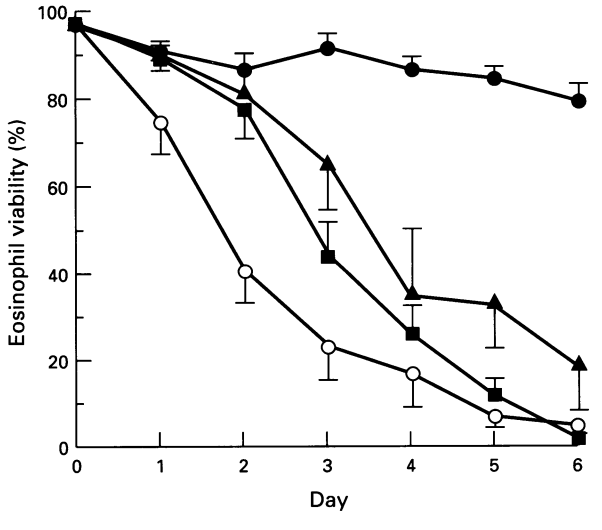

Figure 7 Time course of survival of eosinophils cultured in medium alone (○), 1 ng ml^{-1} GM-CSF (●), 10 ng ml^{-1} cholera toxin (■) and 10^{-3}M dibutyryl cyclic AMP (▲). Each point is the mean ± s.e.mean of 6 experiments.

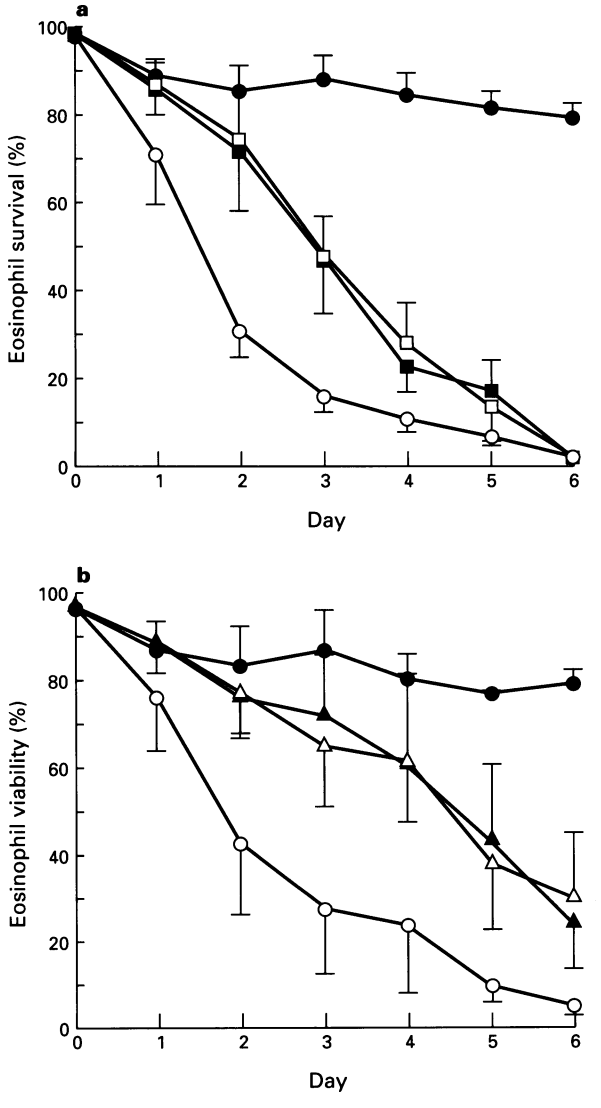

Figure 8 Time course of survival of eosinophils cultured in medium alone (○), 1 ng ml^{-1} GM-CSF (●) and: (a) Cholera toxin (10 ng ml^{-1}) alone (□) and cholera toxin + H89 (10^{-6}M) (■); (b) dibutyryl cyclic AMP (10^{-3}M) alone (△) and dibutyryl cyclic AMP + H89 (10^{-6}M) (▲). Each point is the mean ± s.e.mean of 3 experiments.

drolysable, cell permeable analogue of cyclic AMP, binds to and activates protein kinase A (PKA) directly. The observation that H89, an inhibitor of PKA, prevented the ability of cholera toxin to promote cell death suggests that these agents work via the cyclic AMP/PKA cascade.

Cyclic AMP has previously been shown to have an inhibitory effect on cell function. For instance, elevation of cyclic AMP is associated with the inhibition of physiological responses in leukocytes including exocytotic processes (Bourne *et al.*, 1972), lysosomal enzyme release (Ignarro & George, 1974; Nourshargh & Hoult, 1986), proliferative responses (Smith *et al.*, 1971), phagocytosis (Cox & Karnovsky, 1973) and chemotaxis (Rivkin *et al.*, 1975; Harvath *et al.*, 1991). Biochemical events such as superoxide anion production (Sedgwick *et al.*, 1985; Nielson 1987; Dent *et al.*, 1991; 1994), inositol phospholipid metabolism (Takenawa *et al.*, 1986; Kato *et al.*, 1986) and arachidonic acid release (Takenawa *et al.*, 1986) are also inhibited by increases in cyclic AMP. Cyclic AMP has also been shown to regulate degranulation in human eosinophils (Kita *et al.*, 1991). The present observation that cyclic AMP induces cell death suggests a critical role for this second messenger in the control of cellular turnover in human eosinophils. Evidence for apoptosis was assessed by examination of DNA fragmentation on agarose gel electrophoresis. Previous studies have shown that when eosinophils undergo apoptotic death due to cytokine withdrawal, the DNA is cleaved into oligonucleosome length fragments by the action of endonucleases, exhibiting a 'ladder' pattern when run on agarose gels and that GM-CSF is able to prevent this breakdown of DNA. The ability of cholera toxin and dibutyryl cyclic AMP to overcome the protective effect of GM-CSF on DNA fragmentation suggests that cyclic AMP is able to interfere with the anti-apoptotic signals induced by GM-CSF.

The naturally occurring plant extract, forskolin, is another agent that is reported to elevate cyclic AMP levels, by directly activating adenylyl cyclase, in both membranes and intact cells (Seamon *et al.*, 1981), including thymocytes (MacConkey *et al.*, 1990), T-lymphocytes (Rothman *et al.*, 1993) and neutrophils (Harvath *et al.*, 1991; Tyagi *et al.*, 1991). We therefore evaluated its capacity to affect cellular viability. Forskolin had no effect on eosinophil survival at concentrations up to 10^{-5}M, and did not alter intracellular cyclic AMP levels measured at 24 h over the same concentration range. The reason for this anomalous result presumably relates to the inability of forskolin to activate PKA in human eosinophils, even at concentrations that produce a marked activation in other cells and tissues. Preliminary evidence has suggested that forskolin does not elevate cyclic AMP levels in guinea-pig eosinophils and human monocytes (Giembycz, unpublished observations). Alternatively, methodology may be responsible for the lack of effect of forskolin on cyclic AMP levels in this study. Previous studies with forskolin have been carried out over relatively short periods of time, with cyclic AMP levels measured over minutes, whereas in our studies we have measured cyclic AMP levels up to 24 h. It is possible that forskolin may increase cyclic AMP rather transiently and that cyclic AMP concentrations have returned to basal levels when measured at 24 h. This would be in contrast to the increase in cyclic AMP produced by either cholera toxin, which induces continual stimulation of adenylyl cyclase, or dibutyryl cyclic AMP which is not hydrolysed by PDE enzymes. Stimulation of cells with either of these agents would therefore result in a sustained increase in cyclic AMP.

Inhibition of cyclic AMP hydrolysis with selective inhibitors of PDE has also been used in various studies as a way of increasing cyclic AMP levels. In eosinophils from several species, cyclic AMP is degraded predominantly, if not exclusively, by one or more members of the PDE4 isoenzyme family (Dent *et al.*, 1991; 1994; Hatzelmann *et al.*, 1995). Consistent with these findings we and others have reported previously that *acute* exposure (less than 30 min) of human and guinea-pig eosinophils to selective inhibitors of PDE4 leads to an increase in the cyclic AMP and an associated inhibition of certain functional responses including the generation of oxygen-derived free radicals and lipid mediators (Dent *et al.*, 1991; 1994; Souness *et al.*, 1991; 1994). Since cyclic AMP-elevating drugs generally suppress the activity of eosinophils (Giembycz & Barnes, 1993) we assessed the effect of a PDE3 and a PDE4 inhibitor (SK&F94120 and rolipram respectively) on GM-CSF-induced eosinophil survival. Contrary to what would be predicted from evidence reported in the literature, rolipram failed to inhibit the survival of human eosinophils maintained by GM-CSF, under conditions where cholera toxin and dibutyryl cyclic AMP were effective. It is possible that a sustained increase in the concentration of cyclic AMP of greater magnitude than that elicited by rolipram is required to oppose the effects of GM-CSF. Another important consideration is that chronic treatment of human eosinophils with rolipram may have stimulated transcription and subsequent translation of one or more of the genes that encode PDE4 isoenzymes. This has been reported in a number of cell types (Schwartz & Passoneau, 1974; Torphy *et al.*, 1992; Monaco *et al.*, 1994) and results in the accelerated hydrolysis of cyclic AMP thereby limiting the duration and magnitude of the cyclic AMP signal. It is noteworthy that induction of PDE4 would not affect the ability of dibutyryl cyclic AMP to promote cell death since it is not a substrate for PDE4. Similarly, cholera toxin, by producing a marked and sustained increase in cyclic AMP, may be largely unaffected by the increase in basal PDE activity.

Since an increase in cyclic AMP levels inhibits eosinophil survival induced by GM-CSF, we have therefore investigated the possibility that GM-CSF may prolong eosinophil survival by either decreasing or preventing increases in intracellular cyclic AMP levels. Nucleotide levels were measured up to 24 h in eosinophils cultured in the absence and presence of GM-CSF. We found that GM-CSF had no effect on cyclic AMP levels during culture and did not attenuate the increase in cyclic AMP produced by cholera toxin. Thus, the mechanism by which GM-CSF prolongs eosinophil survival is not related to detectable reductions in cyclic AMP within the cell. The signal transduction events associated with GM-CSF-induced eosinophil survival remain unclear, although recent reports have suggested the involvement of certain tyrosine kinases in cytokine signalling (van der Bruggen *et al.*, 1993; Yousefi *et al.*, 1994) and these mechanisms require further study.

Our findings suggest that the signal transduction processes involved in GM-CSF-induced eosinophil survival and those governing cell death induced by cyclic AMP are not directly related. Indeed, cholera toxin and dibutyryl cyclic AMP, paradoxically, prolonged eosinophil survival in the absence of GM-CSF, by a mechanism that was insensitive to H89 and therefore may be PKA-independent. This differs from the mechanism for inhibition of GM-CSF-induced cell survival by cyclic AMP, which is PKA-dependent. Although protein kinase A is the principle intracellular receptor for cyclic AMP, there is evidence that cyclic AMP can also activate protein kinase G (PKG) (Lincoln *et al.*, 1990). In our experiments dibutyryl cyclic GMP did not affect eosinophil survival under any condition, indicating that cyclic AMP does not mediate its effects by activation of PKG in human eosinophils. An explanation for the differential effects of cyclic AMP may relate to the kinetics of the eosinophil survival in the absence and presence of GM-CSF. In the presence of GM-CSF inhibition of cell survival was observed after 4 days in culture, after chronic exposure to the cyclic AMP elevating agents. In contrast, the enhancement of cell survival in the absence of GM-CSF is relatively short-lived. The mechanism for cyclic AMP-induced enhanced survival of the human eosinophil is unknown and further work is required. However, it is clear that cyclic AMP exerts opposing effects on the survival of human eosinophils, which is dependent on prior exposure of the cells to GM-CSF.

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