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Differential effect of phosphodiesterase 4 inhibitors on the proliferation of human peripheral blood mononuclear cells from normals and subjects with atopic dermatitis

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1 The aims of this study were to compare the effects of selective inhibitors of the type 3, type 4 and type 5 phosphodiesterase (PDE) isoenzymes on the phytohaemagglutinin (PHA)-stimulated proliferation of human peripheral blood mononuclear cells (HPBM) from normals and subjects with atopic dermatitits (AD).

2 Mononuclear cells were isolated from peripheral venous blood of normals and subjects with AD. A concentration-response curve was carried out with PHA (0.5-5 $\mu g\ ml^{-1}$) and a concentration which produced a submaximal stimulation of proliferation (2 $\mu g\ ml^{-1}$) was selected for further experiments. HPBM (10^5 cells per well) were stimulated with PHA (2 $\mu g\ ml^{-1}$) in the absence or presence of PDE inhibitor (0.01 μM-10 μM) and 24 h later [^{3}H]-thymidine (0.1 μCi per well) was added. Cells were incubated for an additional 24 h period and [^{3}H]-thymidine incorporation measured.

3 The type 4 PDE inhibitors (rolipram, RO 20-1724 and denbufylline) produced a concentration-related inhibition of proliferation of HPBM from normal and AD subjects. The IC_{50} for rolipram was significantly ($P<0.05$) lower in HPBM from AD patients 0.28 μM (95% confidence limits (CL): 0.158-0.499, $n=5$) vs normal subjects 2.6 μM (95% CL: 0.867-7.05, $n=5$, $P<0.05$) as were the IC_{50} values for denbufylline: 0.26 μM (95% CL: 0.152-0.440, $n=5$) vs 1.84 μM (95% CL: 0.467-7.23, $n=5$, $P<0.05$) respectively and RO 20-1724: 1.49 μM (95% CL: 0.61 μM-3.64 μM) vs 6.46 μM (95% CL: 2.03 μM-20.46 μM), respectively.

4 The mixed type 3/4 inhibitors (zardaverine and benzafentrine) produced a concentration-related inhibition of proliferation of HPBM from normal and AD subjects. The IC_{50} value for zardaverine in HPBM from normal subjects: 1.8 μM (95% CL: 0.43 μM-7.85 μM, $n=4$) was similar to that in AD subjects: 1.03 μM (95% CL: 0.48 μM-2.28 μM) as was the IC_{50} value for benzafentrine in normal 3.8 μM (95% CL: 2.45 μM-5.9 μM) and atopic 5.5 μM (95% CL: 3.84 μM-7.78 μM) HPBM. The type 5 PDE inhibitor, zaprinast was ineffective at inhibiting the proliferation of normal HPBM. The type 3 PDE inhibitor, siguazodan only inhibited [^{3}H]-thymidine incorporation at a concentration of 10 μM.

5 These results show that combined inhibition of the type 3 and 4 PDE isoenzymes in HPBM from normal subjects has a greater antiproliferative effect than inhibition of the type 4 isoenzyme alone. In addition these data indicate that the proliferative response of HPBM from AD subjects is more sensitive to PDE 4 inhibition than the proliferation of HPBM from normals.

Keywords: Isoenzyme selective PDE inhibitors; peripheral blood mononuclear cells; atopy

Introduction

Atopic diseases such as bronchial asthma and atopic dermatitis (AD) are characterized by the presence of a localized infiltrate of inflammatory cells including lymphocytes, mast cells, basophils and eosinophils and there is evidence to suggest that activated T-lymphocytes orchestrate this allergic inflammatory response (Azzawi *et al.*, 1992).

Intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) is an important regulator of a variety of lymphocyte functions (Coffey, 1988) and agents which have the ability to elevate cyclic AMP levels in lymphocytes have been demonstrated to possess immunosuppressive properties. A number of groups have shown that such agents are able to suppress lymphocyte blastogenesis (Novogrodsky *et al.*, 1979; Robicsek *et al.*, 1991), proliferation (Mary *et al.*, 1987) and activation (Bourne *et al.*, 1973; Coffey, 1988; Kammer, 1988; Lingk *et al.*, 1990; Meskini *et al.*, 1992; Schudt *et al.*, 1992; Giembycz *et al.*, 1994).

One mechanism by which cyclic AMP may be elevated within cells is by inhibition of the phosphodiesterase (PDE) enzyme. This family of enzymes is presently known to exist in at least 7 different isoenzyme forms (Giembycz & Kelly, 1994) which are characterized by a variety of criteria including their sensitivity to different inhibitors (Nicholson & Shahid, 1993). The predominant PDE isoenzyme in most inflammatory cells is PDE 4 (Giembycz, 1992). However, both the type 3 and 4 PDE isoenzymes have been characterized (Thompson *et al.*, 1976; Takemoto *et al.*, 1978; Robicsek *et al.*, 1991) and are known to control cyclic AMP breakdown in human T-lymphocytes (Schudt *et al.*, 1992).

Previous studies have shown abnormalities of immune function in AD; for example one study demonstrated an excessive histamine release by atopic basophils (Butler *et al.*, 1983), and another demonstrated hyper IgE synthesis by mononuclear cells from AD patients (Cooper *et al.*, 1985). It has been suggested that these differences may be due to defects in the cyclic nucleotide regulatory system. Indeed there is evidence to suggest that cyclic AMP hydrolytic activity is increased in mononuclear leukocytes from patients with atopic disease (Grewe *et al.*, 1982) which has been attributed to an elevated cyclic AMP specific PDE activity (Butler *et al.*, 1983;

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Holden *et al.*, 1986; Sawai *et al.*, 1995). Moreover the more active cyclic AMP-PDE isoform in atopic leukocytes has been demonstrated to have a greater sensitivity to inhibition by the type IV PDE inhibitor, RO 20-1724 than the PDE in normal leukocytes (Giustina *et al.*, 1984).

In the present study we have examined the ability of a range of isoenzyme selective PDE inhibitors to inhibit the PHA-stimulated proliferation of human peripheral blood mononuclear cells (HPBM) from normal subjects and patients with AD.

Part of this work has been presented to the British Pharmacological Society (Banner & Page, 1994).

Methods

Subjects

Normal healthy subjects who had no history of asthma, allergic rhinitis or AD were selected for this study. In addition a separate group of patients with active atopic dermatitis whose only existing medication was topical corticosteroids were also chosen. This study was approved by the Ethics Committees of King's College Hospital and the Chelsea & Westminster Hospital.

Preparation of human peripheral blood mononuclear cells

For each experiment 25 ml of peripheral venous blood was drawn from a single volunteer and collected in tubes coated with the anti-coagulant ethylenediamine tetraacetate (EDTA). The blood was diluted with 0.9% NaCl (15 ml blood:25 ml NaCl) and added to a leucosep tube (Poly Labo, Paris) containing 15 ml Histopaque (density = 1.077 ± 0.001 g ml^{-1}) beneath a porous barrier and centrifuged for 10 min at 1100 *g* (20°C). The plasma was then aspirated and mononuclear cells were collected with a pasteur pipette. Sterile NaCl (5 ml of 0.9%) was added to the recovered mononuclear cell fraction and it was centrifuged for 10 min at 1100 *g* (0°C). The supernatant was then aspirated and the cell pellet re-suspended in 5 ml sterile NaCl and centrifuged for a further 10 min. This was repeated a further time and cells were then re-suspended in Spinner modified minimal essential medium (SMEM) containing L-glutamine (2 mM), non-essential amino acids (1% v/v), sodium pyruvate (1 mM), penicillin (100 u ml^{-1}), streptomycin (100 μg ml^{-1}) and HEPES (20 mM). Total cell counts were performed with an improved Neubauer haemocytometer and cell viability was assessed by trypan blue exclusion. The volume of cells was then adjusted to 10^6 cells ml^{-1} in culture media with 10% Ultroser. Approximately $1-1.5 \times 10^6$ cells were routinely obtained per ml of blood and cell viability was always >95%. Platelet contamination was less than one platelet per nucleated cell.

Preparation of plate and PDE inhibitors

PDE inhibitors were dissolved in dimethylsulphoxide (DMSO) and stock solutions were made at a concentration of 10^{-2} M. Dilutions were made in SMEM culture media. HPBM (10^5) were added to each well of a 96 well plate in a volume of 100 μl and 50 μl of PHA was added to each well at a final concentration of 2 μg ml^{-1}; 50 μl of PDE inhibitor or vehicle was then added to each well. Control wells received 50 μl culture media and 50 μl of the vehicle, DMSO which was at a final concentration in each well of no greater than 0.1%. This was the final concentration that DMSO would be at in cells that were treated with the highest concentration, 10 μM, of PDE inhibitor. This concentration of DMSO was found to have no significant effect on cell proliferation. The cells were then incubated at 37°C in a 95% air, 5% CO_2 atmosphere.

Assessment of proliferation

Twenty four hours later [^{3}H]-thymidine (0.1 μCi) was added to each well diluted in SMEM media and the cells were incubated for a further 24 h period. Cells were then harvested onto glass fibre filters using a cell harvester (ICN Flow, Buckinghamshire) and counted in a scintillation counter.

Statistical tests

Each drug was examined on blood samples from four or five volunteers and each concentration of PDE inhibitor examined in triplicate. Geometric means were calculated together with 95% confidence limits. Data are expressed as means $\pm$ s.e.mean. Drug-treated cells were compared with vehicle-treated cells by Dunnett's test.

Materials

SMEM, L-glutamine, non-essential amino acids (10×), sodium pyruvate, penicillin streptomycin (Gibco), HEPES, dimethylsulphoxide, Histopaque, trypan blue, chromotrope 2R, Erhlichs Haematoxylin (Sigma diagnostics, Dorset), Ultroser (Jones Chromatography, Mid Glamorgan, U.K.), phytohaemagglutinin (PHA), leucosep tubes (Poly Labo, Paris), [6-^{3}H]-thymidine (Amersham International, Buckinghamshire). The following drugs were also used: vinpocetine (Gedeon Richter Ltd, Budapest, Hungary), siguazodan (SmithKline Beecham Pharmaceuticals, King of Prussia, PA, U.S.A.), rolipram (ZK 62711) (Schering AG, Berlin, Germany), denbufylline (SmithKline Beecham Pharmaceuticals), RO 20-1724 (4-(3-butoxy-4-methoxy-benzyl)-2-imidazolidinone; Calbiochem), zardaverine (B 842-90) (Forschungslaboratorien Byk Gulden, Germany), benzafentrine (Sandoz), zaprinast (M&B 22,948) (Rhone-Poulenc Rorer, Dagenham, England).

Results

PHA produced a concentration-related stimulation of [^{3}H]-thymidine incorporation of human peripheral blood mononuclear cells (HPBM) from normal subjects and patients with AD. The response in each case was bell-shaped. There was no significant difference in EC_{50} values between atopic and normal HPBM. Likewise the maximum proliferative responses of normal and atopic HPBM were similar. A concentration of 2 μg ml^{-1} produced a submaximal (EC_{90}) stimulation of [^{3}H]-thymidine incorporation in normal (5005 ± 1607 d.p.m.) and AD (8300 ± 868 d.p.m.) HPBM compared with basal levels of 148 ± 18 d.p.m. and 400 ± 57, respectively. There was no signifiant difference in the basal incorporation of [^{3}H]-thymidine between atopic and normal HPBM. Similarly there was no significant difference in the PHA (2 μg ml^{-1})-stimulated proliferative response obtained from normal and atopic HPBM. This concentration of PHA (2 μg ml^{-1}) was selected for subsequent experiments with PDE inhibitors.

The type 3 PDE isoenzyme inhibitor, siguazodan had no effect on normal or atopic HPBM proliferation over the concentration range 0.01 μM–3 μM, but significantly inhibited proliferation at a concentration of 10 μM ($P<0.05$). At this concentration, approximately 25% inhibition of HPBM proliferation was observed (Figure 1).

In contrast the type 4 PDE inhibitors (rolipram, denbufylline and RO 20-1724) produced a concentration-related inhibition of the proliferation of HPBM from both normal and AD subjects (Figure 2a, b and c respectively), although the HPBM from AD patients were more sensitive to PDE IV inhibition. The IC_{50} for rolipram was significantly lower in HPBM from AD patients 0.28 μM (95% confidence limits (CL): 0.158–0.499, $n=5$) vs normal subjects 2.6 μM (95% CL: 0.867–7.05, $n=5$, $P<0.05$) (Figure 2) as were the IC_{50} values for denbufylline: 0.26 μM (95% CL: 0.152–0.440, $n=5$) vs 1.84 μM (95% CL: 0.467–7.23, $n=5$, $P<0.05$) respectively (Figure 2) and RO 20-1724: 1.49 μM (95% CL: 0.61 μM–3.64 μM) vs 6.46 μM (95% CL: 2.03 μM–20.46 μM) respectively ($P<0.05$). In addition rolipram (10 μM), denbufylline (10 μM) and RO 20-1724 (10 μM) almost completely inhibited

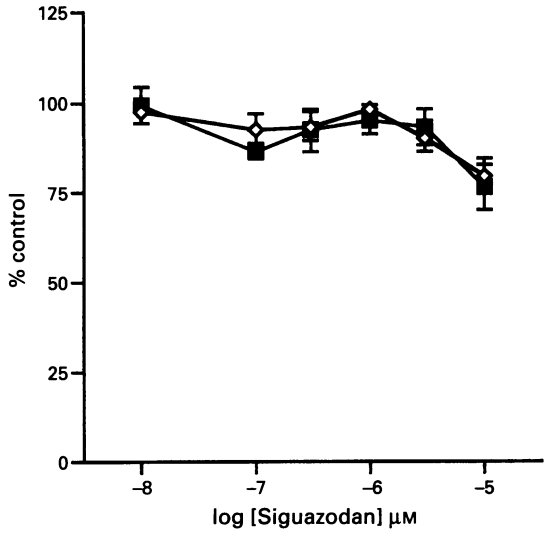

Figure 1 Effect of siguazodan on the phytohaemagglutinin (PHA, 2 $\mu g ml^{-1}$) stimulated proliferation of human peripheral blood mononuclear cells (HPBM) from normals and subjects with atopic dermatitis. Data are expressed as % of the values obtained for cells stimulated with PHA in the absence of drug. (■) Atopic HPBM and (△) normal HPBM. Values are mean ± s.e.mean ($n=4$ per group).

the PHA-stimulated proliferation of HPBM from AD subjects, whereas the same concentrations of rolipram, RO 20-1724 and denbufylline inhibited the proliferation of HPBM from normal subjects by only 60%.

The mixed type 3/4 PDE isoenzyme inhibitor, zardaverine also inhibited normal and atopic HPBM proliferation in a concentration-dependent manner (Figure 3a). At the highest concentration examined, 10 μM, zardaverine almost completely reduced both normal and atopic HPBM proliferation to basal levels. The IC_{50} for zardaverine in HPBM from normal subjects was 1.8 μM (95% CL: 0.43 μM – 7.85 μM) compared with 1.03 μM, (95% CL: 0.48 μM – 2.28 μM) in atopic patients. The mixed type 3/4 inhibitor, benzafentrine also produced a concentration-related inhibition of HPBM from both normal and atopic subjects with IC_{50} values of 3.8 μM (95% CL: 2.45 μM – 5.9 μM) in normal and 5.5 μM (95% CL: 3.84 μM – 7.78 μM) in atopic HPBM (Figure 3b).

Zaprinast had no effect on the PHA-stimulated proliferation of HPBM from normal subjects over the concentration range examined (Figure 4).

Discussion

An increase in cyclic AMP levels via the inhibition of PDE has been shown to inhibit the activation of a variety of cells which are thought to be involved in the inflammatory response including macrophages, eosinophils and T-lymphocytes (Torphy & Undem, 1991). The type 4 PDE isoenzyme appears to be the main PDE isoenzyme present in inflammatory cells and has been characterized in T-lymphocytes, eosinophils, neutrophils, monocytes, basophils, mast cells and endothelial cells (Souness *et al.*, 1991; Giembycz, 1992), although the type 3 PDE isoenzyme has also been identified in human peripheral blood lymphocytes (Thompson *et al.*, 1976; Robicsek *et al.*, 1991).

The present results show that the type 5 PDE inhibitor, zaprinast had no effect on the PHA-stimulated proliferation of HPBM from normal subjects suggesting that the type 5 isoenzyme is not involved in the control of proliferation. The type 3 isoenzyme inhibitor, siguazodan only inhibited the proliferation of HPBM from both normals and AD subjects at the highest concentration tested, 10 μM. Since a high concentration of this drug was required to inhibit proliferation, it is conceivable that these antiproliferative effects may be due to an action on PDE isoenzymes other than the type 3 PDE.

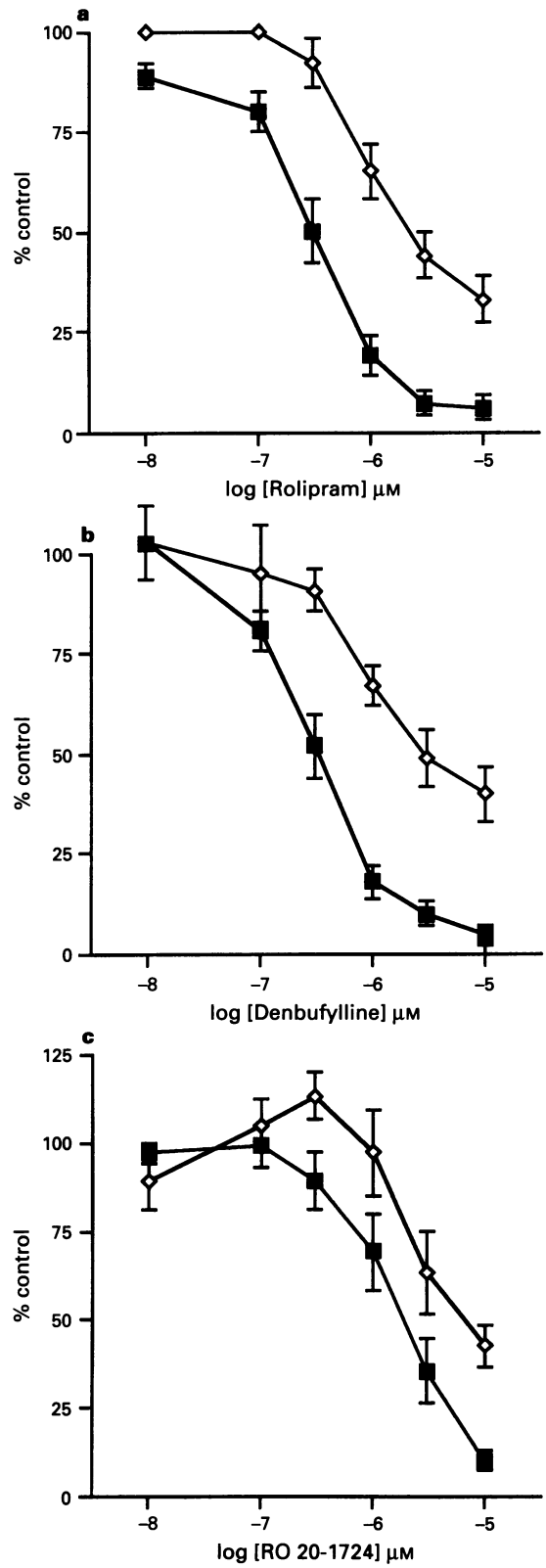

Figure 2 Effect of (a) rolipram, (b) denbufylline and (c) RO 20-1724 on the phytohaemagglutinin (PHA, 2 $\mu g ml^{-1}$) stimulated proliferation of human peripheral blood mononuclear cells (HPBM) from normals and subjects with atopic dermatitis. Data are expressed as % of the values obtained for cells stimulated with PHA in the absence of drug. (■) Atopic HPBM and (◇) normal HPBM. Values are mean ± s.e.mean ($n=5$ per group).

The type 4 PDE inhibitors, rolipram, RO 20-1724 and denbufylline produced a concentration-related inhibition of PHA-stimulated HPBM proliferation from normal subjects. This is consistent with the findings of other groups that have

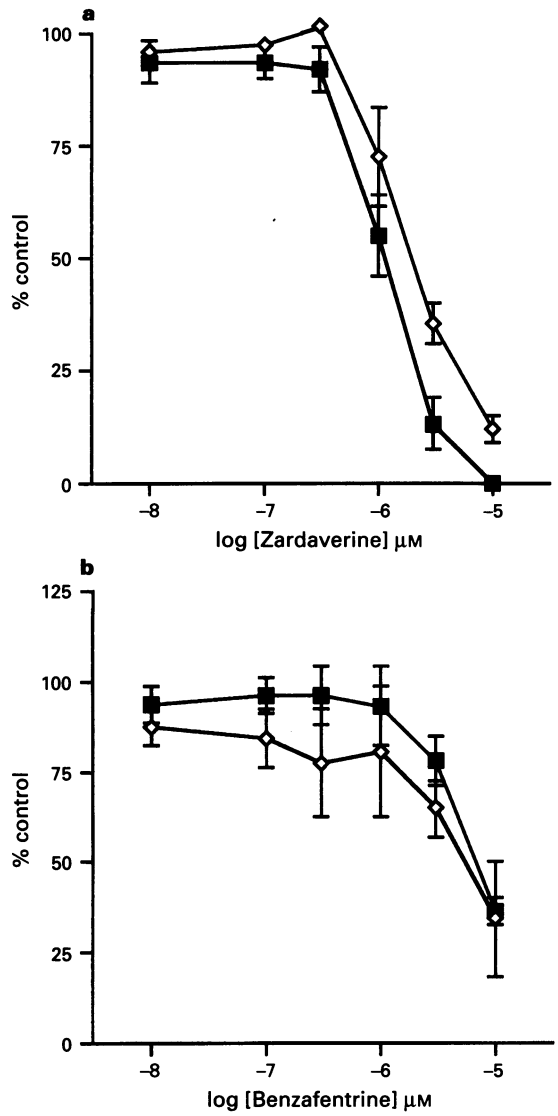

Figure 3 Effect of (a) zardaverine and (b) benzafentrine on the phytohaemagglutinin (PHA, $2\,\mu g\,ml^{-1}$) stimulated proliferation of human peripheral blood mononuclear cells (HPBM) from normals and subjects with atopic dermatitis. Data are expressed as % of the values obtained for cells stimulated with PHA in the absence of drug. (■) Atopic HPBM and (◇) normal HPBM. Values are mean ± s.e.mean ($n=5$ per group).

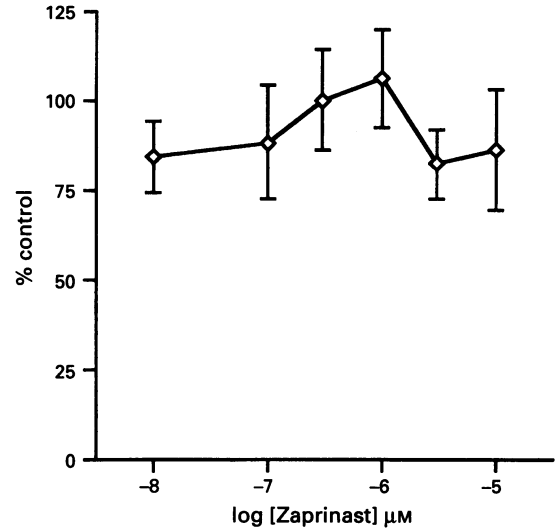

Figure 4 Effect of zaprinast on the phytohaemagglutinin (PHA, $2\,\mu g\,ml^{-1}$) stimulated proliferation of human peripheral blood mononuclear cells (HPBM) from normal subjects. Data are expressed as % of the values obtained for cells stimulated with PHA in the absence of drug. Values are mean ± s.e.mean ($n=3$ per group).

demonstrated an inhibitory effect of type 4 PDE inhibitors on PHA-stimulated T-lymphocyte (Robicsek *et al.*, 1991) and HPBM (Pazdur *et al.*, 1980) proliferation. It has also previously been shown that type IV PDE inhibitors can inhibit anti-CD3 stimulated lymphocyte proliferation (Schudt *et al.*, 1992).

The type 4 PDE inhibitors, rolipram, RO 20-1724 and denbufylline produced approximately a 60% inhibition of the proliferative response of HPBM from normal subjects at 10 μM but were unable to inhibit proliferation completely. In contrast the mixed type 3/4 PDE inhibitor, zardaverine almost completely suppressed proliferation at a concentration of 10 μM. This would suggest that combined inhibition of both the type 3 and type 4 PDE isoenzymes is more effective at inhibiting PHA-stimulated proliferation than inhibition of the type 4 PDE isoenzyme alone. These findings are similar to two studies which demonstrated that combined inhibition of the type 3 and 4 isoenzymes was more effective at inhibiting proliferation than inhibition of either isoenzyme alone (Robicsek *et al.*, 1991; Schudt *et al.*, 1992). However the studies described above have just examined the effect of PDE 3, 4 and mixed PDE 3/4 inhibitors on HPBM from normal healthy subjects.

In our study we have compared the effect of PDE inhibitors on the proliferation of HPBM from both normals and subjects with AD. The PDE 4 inhibitors produced a concentration-related inhibition of atopic HPBM proliferation. However, we found that the PDE 4 inhibitors were more effective at inhibiting atopic HPBM proliferation compared with normal HPBM proliferation. There is evidence from a number of studies that cyclic AMP PDE activity is elevated in mononuclear leukocytes from atopic subjects compared with normal subjects (Grewe *et al.*, 1982; Butler *et al.*, 1983; Holden *et al.*, 1986; Sawai *et al.*, 1995). However, it is difficult to predict whether this, in itself, would render cells more or less susceptible to the effects of PDE 4 inhibitors. Moreover, there is little evidence from the present study to suggest that PDE 4 activity was upregulated in the cells from atopics. If it were, and if this were relevant, one would have expected to find PHA-stimulated atopic HPBM proliferation to be elevated (before addition of PDE inhibitors). Although this appeared to be the case as a trend, there was actually no significant difference between atopics and normals in this regard. As an alternative and preferable explanation, since the type 4 PDE inhibitor, RO 20-1724, has been found to be more effective at inhibiting the activity of the cyclic AMP PDE in cells from atopics compared with normals (Chan & Hanifin, 1993) this implies that some change in PDE isoenzyme profile may make atopics intrinsically more susceptible to the effects of PDE 4 inhibitors than normal subjects. Indeed, there is evidence to suggest that a unique form of PDE is expressed in monocytes from AD patients (Chan *et al.*, 1993).

In contrast to the increased effectiveness of the type 4 PDE inhibitors at inhibiting the proliferation of AD cells versus normals, the mixed 3/4 PDE inhibitors were equally effective in AD and normal cells. The reasons for this are not unclear. We can speculate that no further inhibition of proliferation by zardaverine can occur in AD cells, because in normal cells inhibition of type 3 and 4 PDE results in complete inhibition of proliferation. Another explanation is that in atopic cells there is a downregulation of the type 3 PDE isoenzyme and/or a corresponding elevation in PDE 4 activity. Alternatively rolipram, denbufylline and RO 20-1724 may have a higher affinity than zardaverine for the PDE enzyme in AD HPBM than in normals. Moreover, if differences exist between atopics and

normal subjects in terms of T cells and monocytes in their PBMC samples then this might further complicate matters. These issues might be resolved in part by the use of pure populations of T cells or monocytes, and by comparing the actions of selective PDE 3 and PDE 4 inhibitors with the effects of different ratios of combinations of PDE 3 and PDE 4 inhibitors.

It is not clear at which stage in the proliferation process the PDE inhibitors are acting to inhibit HPBM proliferation. T cell activation and proliferation requires two initial signals. PHA stimulates T cells to pass from the G_0 phase to the G_1 phase of the cell cycle and to synthesize protein (Hadden, 1988). A second signal, interleukin 1 (IL-1) which is produced by adherent accessory cells is required to initiate DNA synthesis and acts on primed T-lymphocytes to induce the production of interleukin 2 (IL-2) and the appearance of IL-2 receptors (Smith & Cantrell, 1985). Activated T-lymphocytes then proliferate in response to IL-2 (Toribio *et al.*, 1989).

The PDE inhibitors could therefore either be acting at the level of the accessory cells to block IL-1 release or they could be having a direct effect on the lymphocytes to inhibit proliferation. The non-selective PDE inhibitor theophylline has been shown to suppress human alveolar macrophage respiratory burst through PDE inhibition (Dent *et al.*, 1994).

If the drugs are inhibiting proliferation by a direct effect on lymphocytes then there are a number of stages of the proliferation process at which they could be acting. They could be having an inhibitory effect on IL-2 production. There is evidence that an increase in cyclic AMP resulting from inhibition of the type 4 PDE isoenzyme may cause a decrease in TNF, IL-1 and IL-2 generation from monocytes and lymphocytes (Averill & Kammer, 1985; Maschler & Christensen, 1991; Giembycz *et al.*, 1994). A separate study has shown that prostaglandin E_2 (PGE_2) through an elevation of cyclic AMP levels can inhibit IL-2 production from Th_1 murine clones (Betz & Fox, 1991). Alternatively they could be either preventing T-lymphocytes from becoming responsive to IL-2 or they could be having some effect resulting in reduced IL-2 receptor expression.

There is also evidence to suggest from several studies that patients on long term theophylline therapy have an increase in the number and activity of T suppressor cells (Shohat *et al.*, 1983; Fink *et al.*, 1987; Milgrom, 1991). These theophylline sensitive cells have been shown to suppress peripheral blood mononuclear cell proliferation (Lahat *et al.*, 1985). It would therefore also seem plausible that the type 4 PDE inhibitors could be having a stimulatory effect on T suppressor cells. This is of interest given that theophylline has recently been suggested to possess immunomodulatory activity in asthmatic subjects (Ward *et al.*, 1993; Jaffar *et al.*, 1994).

Lastly it is possible that the drugs are having a cytotoxic effect since there is evidence to suggest that high levels of cyclic AMP are cytotoxic. However, since the inhibition by PDE isoenzyme inhibitors did not reduce [^{3}H]-thymidine incorporation to below that in unstimulated cultures this would suggest that cell viability was not being affected.

In conclusion, these results show that combined inhibition of the type 3 and 4 PDE isoenzymes in HPBM from normal subjects using a mixed 3/4 inhibitor has a greater antiproliferative effect than inhibition of the type 4 isoenzyme alone. These data also indicate that the proliferation of HPBM from AD subjects is more sensitive to PDE 4 inhibition than the proliferation of HPBM from normals. This may reflect a difference in PDE isoenzyme profile between cells from normal subjects and AD patients.

K.H.B. is a Medical Research Council Scholar.

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