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SELECTIVE INHIBITION OF THROMBOXANE BIOSYNTHESIS IN HUMAN BLOOD MONONUCLEAR CELLS AND THE EFFECTS ON MITOGEN-STIMULATED LYMPHOCYTE PROLIFERATION

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1 The effects of six imidazole compounds were examined on thromboxane B_2 (TxB_2) and prostaglandin E_2 (PGE_2) production and mitogen-stimulated lymphocyte transformation in human blood mononuclear cells.

2 UK 37248 (4-(2-[IH-imidazol-l-yl]ethoxy)benzoic acid), imidazole and l-methylimidazole selectively inhibited TxB_2 synthesis in a dose-related manner, with corresponding increases in PGE_2 production.

3 Clotrimazole, benzimidazole and 2-methylimidazole preferentially inhibited TxB synthesis but had little effect on PGE_2 production.

4 Clotrimazole and benzimidazole inhibited proliferative responses of the lymphocytes, but UK 37248 and l-methylimidazole did not affect transformation at concentrations which inhibited TxB_2 synthesis to a similar level (over 90%).

5 The results do not support involvement of endogenous TxB_2 in the process of lymphocyte mitogenesis or in the mechanism of the suppressive effects of some TxB_2 synthetase inhibitors.

Introduction

Endogenous prostaglandin E_2 (PGE_2) production is believed to modulate the proliferative response of mitogen-stimulated human lymphocytes. Indomethacin, at concentrations just sufficient to abolish PGE_2 production, enhances [^{3}H]-thymidine ([^{3}H]-TdR) incorporation in response to sub-optimal mitogen stimulation, and this enhancement is readily reversed by exogenous PGE_2 at concentrations that can be generated during *in vitro* culture (Goodwin, Messner & Peake, 1978; Gordon, Henderson & Westwick, 1979). The inhibitory effect of PGE_2 may be mediated via elevation of cyclic adenosine 3′,5′-monophosphate (cyclic AMP), which occurs predominantly in the T_G sub-population (Goodwin, Kaszubowski & Williams, 1979) that are also most responsive to low mitogen concentrations (Moretta, Ferrarini, Mingari, Moretta & Webb, 1976; Lum, Beneviste & Blaese, 1980). However, thromboxane (Tx) detected as TxB_2 is the major cyclo-oxygenase product formed from arachidonic acid by human mononuclear cells (Morley, Bray, Jones, Nugteren & Van Dorp, 1979; Kennedy, Stobo & Goldyne, 1980) and has been proposed to fulfil a more fundamental role in lymphocyte mitogenesis (Kelly, Johnson & Parker, 1979) since selective inhibitors of Tx biosynthesis, such as imidazole (Moncada, Bunting, Mullane, Thorogood & Vane, 1977) also inhibited the proliferative response. Imidazole is a fairly weak inhibitor of Tx production necessitating the use of millimolar concentrations which are known to have other effects, e.g. on cyclic nucleotide metabolism (Hadden, Coffey, Hadden, Lopez-Corrales & Sunshine, 1975) that might complicate the interpretation of the results. Furthermore, as Tx synthesis was inhibited, there was some qualitative evidence that prostaglandin endoperoxides were converted to prostaglandins in larger amounts, and, in view of the established inhibitory role of PGE_2 in such responses, this might also have contributed to the observed effects.

In this study the effects of 6 imidazole analogues were examined on TxB_2 and PGE_2 production by human blood mononuclear cells. Although all the compounds preferentially inhibited TxB_2 production, the specificities differed, based on their effects on PGE_2 production. UK 37248, the most selective, and clotrimazole a less specific representative of these 6 imidazole analogues were chosen because of their high potency as tools for re-examining the role of endogenous Tx biosynthesis in mitogenic responses of human lymphocytes. Taking into account the restricted inhibitory effect of endogenous PGE_2,

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the effects of these imidazole compounds were examined over the complete phytohaemagglutinin (PHA) dose-response curve, and at near optimal responses to three other T-cell mitogens (pokeweed, concanavalin A and phorbol myristic acetate).

Methods

Peripheral venous blood from apparently healthy volunteers, who were receiving no medication at the time of study, was collected aseptically via 16-gauge siliconized cannulae. Defibrinated blood was diluted 3:2 (v/v) with Iscove's modified Dulbecco's medium (Gibco) containing antibiotics (100 units penicillin/ml and 100 μg streptomycin/ml, Gibco) and aliquots (18 ml) were layered onto Ficoll-Paque (8 ml, Pharmacia) in sterile, disposable plastic universal containers (Sterilin). Following centrifugation at 450 *g* for 40 min at room temperature, the mononuclear cell-rich interface was removed and the cells were washed twice with Iscove's medium.

Aliquots of cell suspension containing 5×10^5 viable leukocytes were added to 12 × 75 mm sterile, plastic tubes (Falcon) and incubated with or without drugs for 48 h at 37°C in humidified 5% CO_2 atmosphere. PGE_2 and TxB_2 content of cell-free supernatant fluids were estimated by radioimmunoassay using previously described antisera (Bray & Gordon, 1978; Brodie, Hensby, Parke & Gordon, 1980).

Aliquots of cell suspension containing 2×10^5 viable leukocytes were added to 96-well (flat-bottomed type) microtitre plates. Drugs and mitogens in 20 μl volumes of Iscove's medium were added as described at the beginning of culture to give a final volume of 200 μl medium containing 10% heat-inactivated (56°C for 30 min) foetal calf serum (Gibco) per well. The plates were incubated for 72 h at 37°C in humidified 5% CO_2 atmosphere. [^{3}H]-TdR (0.2 μCi in 20 μl, 2 Ci/mmol, Radiochemical Centre, Amersham) was added for the final 18 h of incubation and the content of each well was then collected on Whatman GF/A filter paper using a multiple automated cell harvester. [^{3}H]-TdR incorporation (in counts/min) was measured using standard scintillation counting techniques.

Materials

Purified phytohaemagglutinin (PHA, Wellcome), concanavalin A (Sigma) and pokeweed mitogen (Sigma) dissolved in Iscove's medium and phorbol myristic acetate (Sigma) dissolved in acetone all at 1 mg/ml were stored at −20°C and diluted as required in Iscove's medium on the day of use.

Imidazole (grade III, Sigma) and l-methylimidazole (Sigma) were dissolved directly in Iscove's medium; benzimidazole (Sigma), UK 37248 (4-(2-[IH-imidazol-l-yl]ethoxy)benzoic acid, Pfizer), and clotrimazole (l-(l-*o*-chlorophenyl-l,l-diphenylmethyl)imidazole, Bayer) were dissolved in dilute HCl and further diluted in Iscove's medium; 2-methyl-imidazole (Sigma) was dissolved in dilute NaOH and diluted further in Iscove's medium. All drug solutions were freshly prepared immediately before use and sterilized by membrane filtration (0.22 μm Millex).

Results

Effects of imidazole compounds on prostaglandin E_2 and thromboxane B_2 production by human blood mononuclear cells

In agreement with previous studies, TxB_2 was produced in greater amounts than PGE_2 by human blood mononuclear cells, the observed ratio being 4:1 (range 1.6–8.0 in 5 experiments). Imidazole (3–300 μg/ml) selectively inhibited TxB_2 production in dose-related manner (Figure 1a) with an IC_{50} of 17 μg/ml (range 6.5–37.5 in 5 experiments), whereas PGE_2 production was not inhibited but increased as TxB_2 decreased. Figure 1a also illustrates the qualitatively similar effects of UK 37248 and l-methylimidazole on PGE_2 and TxB_2 production. UK 37248 was most potent (IC_{50} 0.012 μg/ml) of these compounds, and it is noteworthy that even at a concentration of 30 μg/ml there was no diminution of the elevated PGE_2 production, although l-methylimidazole (300 μg/ml) caused some decrease in PGE_2 production. In contrast three compounds, clotrimazole, benzimidazole and 2-methyl-imidazole preferentially inhibited TxB_2 production (Figure 1b), but the increase in PGE_2 production was limited to approximately two fold and therefore did not fully compensate for the decrease in TxB_2 synthesis. Clotrimazole (IC_{50} 0.07 μg/ml) was the most potent of these compounds in inhibiting TxB_2 production but also decreased PGE_2 output at a concentration of 30 μg/ml.

Effects of imidazole compounds on mitogen-stimulated [^{3}H]-thymidine incorporation by human blood mononuclear cells

When UK 37248 and clotrimazole, the most potent compounds as inhibitors of Tx biosynthesis, and representative of both of the activity profiles noted, were examined for effects on [^{3}H]-TdR incorporation into mononuclear cells stimulated with an optimal PHA concentration, only clotrimazole (3–30 μg/ml) inhibited the response (Figure 2) although it was confirmed in samples withdrawn from the micro-cultures

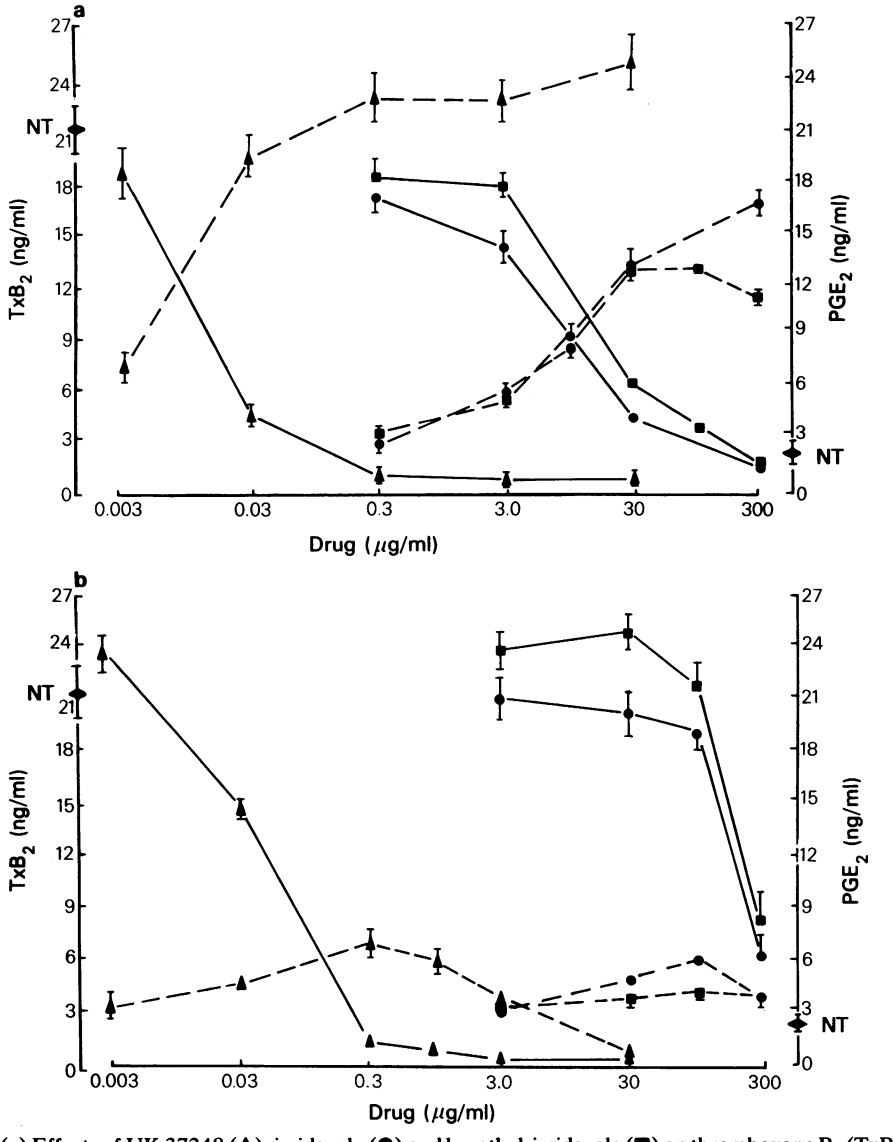

Figure 1 (a) Effects of UK 37248 (▲), imidazole (●) and l-methyl-imidazole (■) on thromboxane B_2 (TxB_2, solid lines) and prostaglandin E_2 (PGE_2, broken lines) production by cultured mononuclear cells. Each value is the mean (vertical lines show s.e.mean) of 3 replicate samples from a single experiment, for which the control TxB_2 and PGE_2 production by untreated cells (NT mean ± s.e.mean of 9 replicates) is indicated. A similar pattern of activity was observed for each compound in 2 separate experiments. (b) Effects of clotrimazole (▲) benzimidazole (■) and 2-methylimidazole (●) on TxB_2 (solid lines) and PGE_2 (broken lines) production by cultured mononuclear cells. Data obtained in the same experiments as shown in (a).

immediately before [^{3}H]-TdR addition, that both compounds inhibited by up to 96% endogenous TxB_2 production.

Figures 3a and 3b illustrate the effects of all six imidazole compounds tested at selected concentrations on the PHA dose-response curve. While UK 37248, imidazole and l-methyl-imidazole partially inhibited (by up to 50%) the responses to sub-optimal PHA (0.02–0.1 μg/ml) stimulation, there was no inhibition of optimal responses (0.5–2.0 μg PHA/ml). On the other hand, clotrimazole and benzimidazole were effective inhibitors of both sub-optimal and optimal PHA responses. Benzimidazole (300 μg/ml) was more effective than

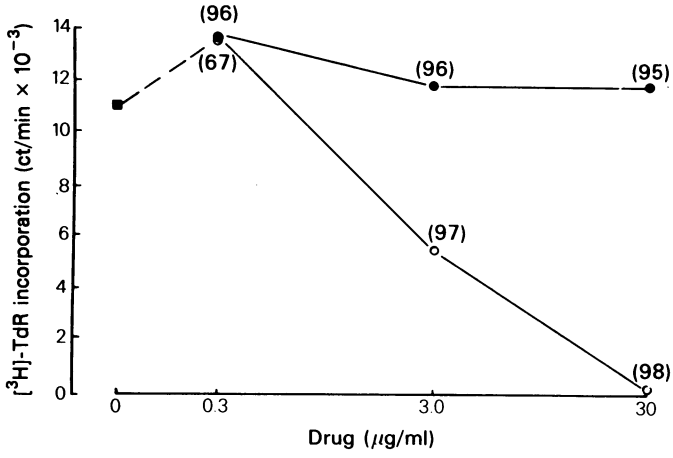

Figure 2 Effects of clotrimazole (O) and UK 37248 (●) on phytohaemagglutinin (PHA)-stimulated (2.0 μg/ml) [^{3}H]-thymidine [^{3}H]-TdR) incorporation by cultured mononuclear cells. Figures in parentheses are the % inhibition of TxB_2 production compared with non drug treated control samples. Each value is the mean of 5 replicate samples from a single representative experiment.

clotrimazole (3 μg/ml) in inhibiting [^{3}H]-TdR incorporation, although less effective in inhibiting TxB_2 production. 2-Methylimidazole (300 μg/ml) and clotrimazole (0.3 μg/ml) which partially inhibited TxB_2 production, as did benzimidazole (300 μg/ml), failed to inhibit [^{3}H]-TdR incorporation significantly, unlike benzimidazole (300 μg/ml).

Clotrimazole (3 μg/ml) also partially inhibited [^{3}H]-TdR incorporation stimulated by three other mitogens (concanavalin A, pokeweed and phorbol myristic acetate, Figure 4) at near optimal concentrations. However, none of the other compounds were effective, at the concentrations tested, in these experiments.

Discussion

These results do not support the proposal (Kelly *et al.*, 1979) that endogenous Tx production by human mononuclear cells has a crucial role in lymphocyte proliferation. In these experiments we distinguished two patterns of activities in the selective Tx synthesis inhibition amongst imidazole compounds, depending on their concomitant effect on the generation of PGE_2 which is the other major cyclo-oxygenation product of arachidonic acid formed by mononuclear cells (Morley *et al.*, 1979; Kennedy *et al.*, 1980). Imidazole, l-methylimidazole and UK 37248 which allowed more or less corresponding increases in PGE_2 production as TxB_2 synthesis decreased, thus indicating that the generation of prostaglandin endoperoxides was little affected, did not depress [^{3}H]-TdR incorporation in response to optimal mitogenic stimulation at concentrations sufficient to inhibit by 95% endogenous Tx production. Thus, the role of changes in both Tx and PGE_2 must be considered.

Endogenously-produced PGE_2 is believed to function as a negative-feedback regulator of lymphocyte mitogenesis when stimulated by concentrations of mitogens that produce sub-optimal responses, e.g. PHA 0.1 μg/ml shown in Figure 3. This is based on the observations (Goodwin *et al.*, 1978; Gordon *et al.*, 1979) that under these conditions indomethacin at low concentrations (1–2 μg/ml) enhances [^{3}H]-TdR incorporation and that this effect is readily reversed by exogenously added PGE_2. Higher concentrations of indomethacin (> 10 μg/ml), which exceed the amount required to abolish detectable cyclo-oxygenase activity of mononuclear cells, are known to inhibit lymphocyte proliferation at all levels of mitogen stimulation. In our experiments, imidazole (300 μg/ml) partially inhibited [^{3}H]-TdR incorporation at sub-optimal PHA stimulation and it is conceivable that elevated PGE_2 production contributed to this effect. However, UK 37248 (0.3–3.0 μg/ml) which inhibited TxB_2 synthesis more effectively and produced a greater increase in PGE_2 generation, was less inhibitory on [^{3}H]-TdR incorporation thus casting doubt on the involvement of either TxB_2 or PGE_2 in this phenomenon. Kelly *et al.* (1979) found that indomethacin did not reverse the inhibition by imidazole of [^{3}H]-TdR incorporation but the concentration of indomethacin used (10 μg/ml) was also inhibitory, possibly unrelated to cyclo-oxygenase. Furthermore, their experiments were performed at optimal levels of mitogen stimulation, at which endogenously-produced PGE_2 has

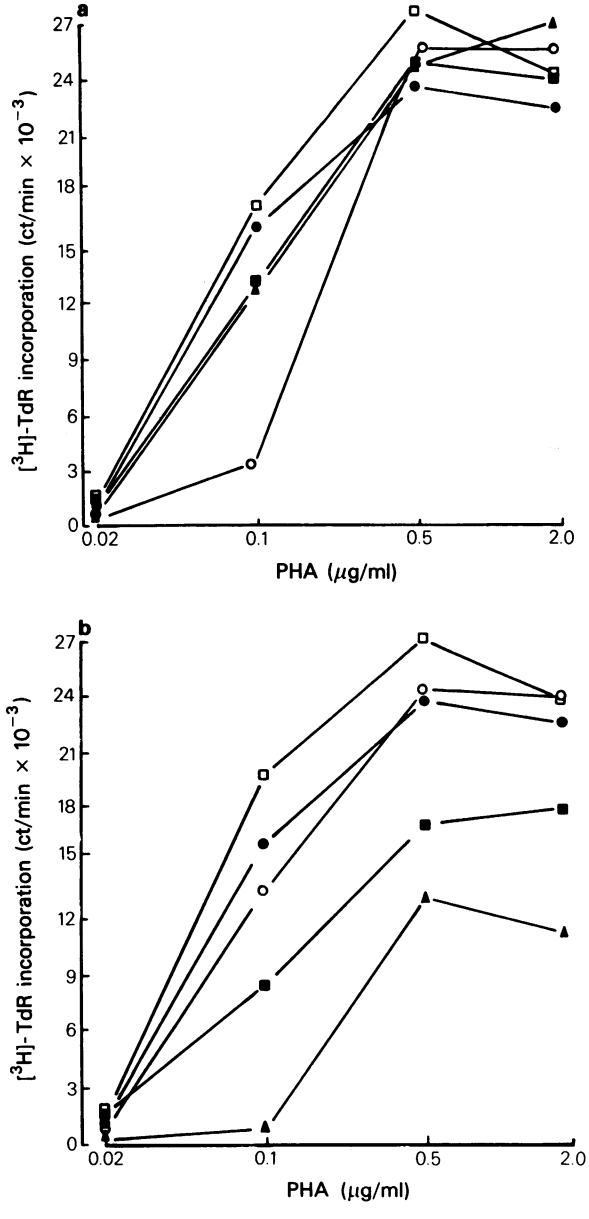

Figure 3 (a) Effects of UK 37248 (0.3 μg/ml, □; 3.0 μg/ml, ■), imidazole (300 μg/ml, ○) and 1-methylimidazole (300 μg/ml, ▲) on phytohaemagglutinin (PHA)-stimulated (PHA alone, ●) [^{3}H]-thymidine ([^{3}H]-TdR) incorporation by cultured mononuclear cells. Each value is the mean of 5 replicate cultures from a single representative experiment. (b) Effects of clotrimazole (0.3 μg/ml, □; 3.0 μg/ml, ■) benzimidazole (300 μg/ml, ▲) and 2-methylimidazole (300 μg/ml, ○) on PHA-stimulated (PHA alone, ●) [^{3}H]-TdR incorporation by cultured mononuclear cells. Data obtained in the same experiment as shown in (a).

been shown to have little inhibitory effect (Goodwin *et al.*, 1978; Gordon *et al.*, 1979). None of the most specific compounds (imidazole, 1-methylimidazole and UK 37248) significantly affected maximum responses to PHA or the three other mitogens examined, further indicating that Tx does not have a fundamental role in this system.

Benzimidazole, 2-methylimidazole and clotrimazole, which allowed no or limited increases in PGE_2 production as TxB_2 synthesis decreased, thus indicating that their action extended to other step(s) in the pathway, were capable of inhibiting [^{3}H]-TdR incorporation both at sub-optimal and optimal levels of stimulation. Clotrimazole was of particular interest because of its reported anti-inflammatory activity in animals and man (Wyburn-Mason, 1976; Otterness & Niblack, 1976). Infiltrating mononuclear cells might be considered as a source of TxB_2 found in synovial effusions of arthritis patients (Brodie *et al.*, 1980). The role of Tx in inflammation is not well understood but the potential for platelet aggregation with release of factors that stimulate mitotic division and other processes in synovial cells (Castor, Ritchie, Scott & Whitney, 1977; Castor, Ritchie, Williams, Scott, Whitney, Myers, Sloan & Anderson, 1979) could be important in the pathogenesis of arthritis. Nevertheless, it is still uncertain why selective Tx synthesis inhibition should be more beneficial than cyclo-oxygenase inhibition, and increases in the production of pro-inflammatory PGE_2 or PGI_2 might be responsible for transient flare-up of symptoms in some patients receiving clotrimazole (Wyburn-Mason, 1976). The inhibition of lymphocyte activation by clotrimazole, observed in these experiments, could also contribute to its anti-arthritic effects, but does not seem attributable to inhibition of Tx synthesis, since comparable effects were not found using UK 37248 which produced a similar degree of inhibition of Tx synthesis. Benzimidazole inhibition of lymphocyte activation may be related to the reported immunosuppressive activities of benzimidazole derivatives (Miller, 1980) but 2-methylimidazole and a low concentration of clotrimazole which produced similar degrees of Tx inhibition failed to affect [^{3}H]-TdR incorporation significantly. Thus, other actions mediate the antiproliferative effects.

Needleman, Bryan, Wychey, Bronson, Eakins, Ferrendelli & Minkes (1977), on the basis of their studies using platelets, cautioned against the indiscriminate use of selective Tx synthesis inhibitors as pharmacological tools, and our results using lymphocytes emphasize this view. A single experiment using a Tx-antagonist, pinane-TxA_2 (Nicolaou, Magolda, Smith, Aharoni, Smith & Lefer, 1979) supported the general conclusion that endogenous Tx production is not of critical importance in lymphocyte mitogenesis (Westwick, Gordon & Nouri, unpublished observa-

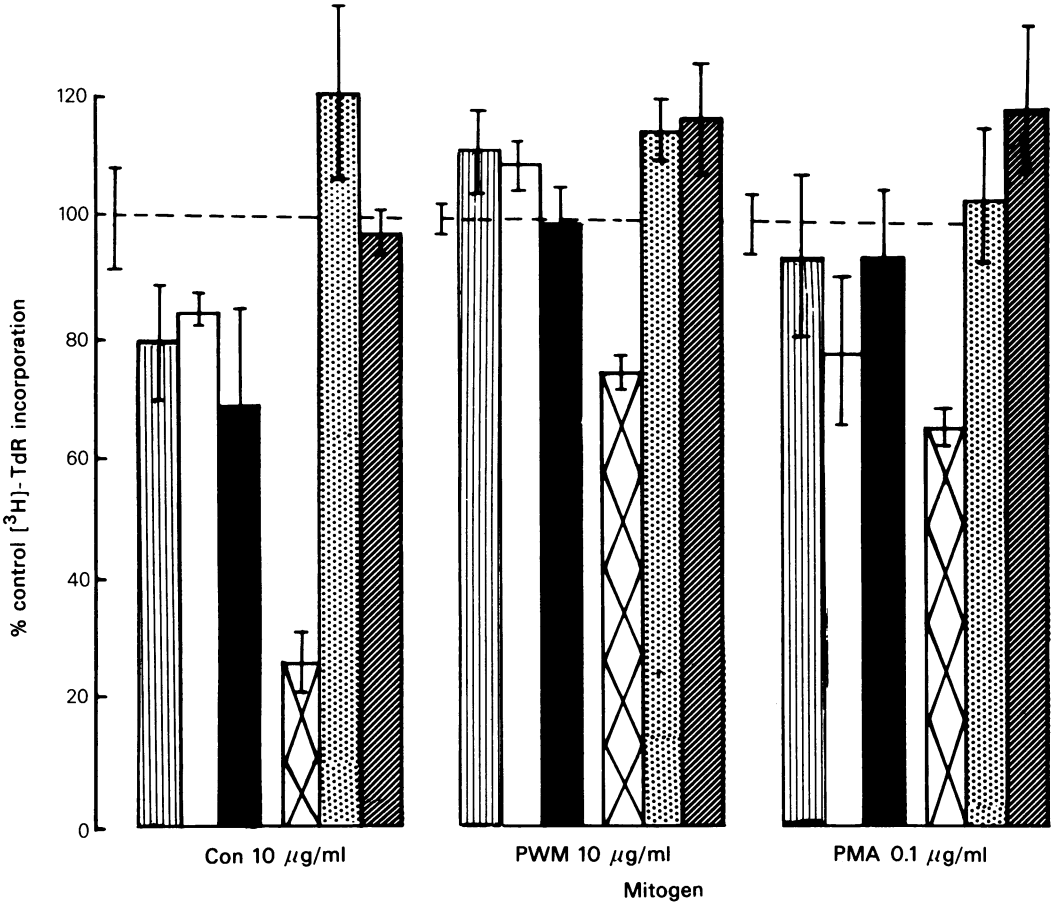

Figure 4 Effects of UK 37248 (3.0 μg/ml, vertical hatching), imidazole (300 μg/ml, open), 1-methylimidazole (300 μg/ml, closed) clotrimazole (3.0 μg/ml, cross-hatching), benzimidazole (300 μg/ml, stippled) and 2-methylimidazole (300 μg/ml, diagonal hatching) on [^{3}H]-thymidine ([^{3}H]-TdR) incorporation by cultured human mononuclear cells stimulated by concanavalin A (Con, 10 μg/ml), pokeweed (PWM, 10 μg/ml) and phorbol myristic acetate (PMA, 0.1 μg/ml). Each value is the mean of 10 replicate cultures from 2 separate experiments; vertical lines show s.e.mean.

tions) but it is difficult to provide an adequate demonstration of effective receptor antagonism in such studies. Although Kelly and co-workers (1979) considered that Tx could be derived from lymphocytes, other investigators (Morley *et al.*, 1979; Kennedy *et al.*, 1980) found that cells of the monocyte/ macrophage series were the major and possibly sole source of cyclo-oxygenase products. Our measurements of TxB_2 and PGE_2 do not discriminate between inhibition of phospholipase and cyclo-oxygenase by the less specific compounds but the effects on phospholipase should be examined since inhibition of this enzyme might also accommodate the actions on lymphocyte proliferation (Hirata, Toyoshima, Axelrod & Waxdal, 1980).

Selective Tx synthetase inhibitors are being developed for their anti-thrombotic potential and UK 37248 recently has been administered to man (Tyler, Saxton & Parry, 1981). If, as was suggested by Kelly *et al.* (1979), Tx was important in lymphocyte mitogenesis, then such compounds might be immunosuppressive which would seriously complicate their long-term usage. Our results, particularly with the most specific compound, UK 37248, predict that this would be unlikely to occur in the prophylactic use of such agents to prevent thrombotic disorders.

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