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# EFFECTS OF PROSTAGLANDINS $E_2$ AND $I_2$ ON HUMAN LYMPHOCYTE TRANSFORMATION IN THE PRESENCE AND ABSENCE OF INHIBITORS OF PROSTAGLANDIN BIOSYNTHESIS

D. GORDON, D.C. HENDERSON,* & J. WESTWICK[1]

Department of Pharmacology & Department of Pathology,* Institute of Basic Medical Sciences, Royal College of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN

**1** The reactivity of human peripheral blood mononuclear cells to phytohaemagglutinin ([$^3$H]-thymidine incorporation) was enhanced in indomethacin- and eicosatetraynoic acid-treated cells compared with untreated cells, from normal volunteers. This suggests that endogenously synthesized prostaglandins have an inhibitory effect during cell preparation and/or culture.

**2** Prostaglandin $E_2$ inhibited [$^3$H]-thymidine incorporation induced by suboptimal phytohaemagglutinin concentrations and had a more potent effect on indomethacin-treated cells than on untreated cells.

**3** Prostaglandin $I_2$ also exhibited an inhibitory effect and, under defined conditions, was more potent than prostaglandin $E_2$ or than prostacyclin which had been allowed to decay at pH 7.4 and 37°C.

**4** These results indicate that, in attempting to define altered lymphocyte reactivity in disease states, the involvement of prostaglandins should be considered both during cell preparation and culture.

## Introduction

Peripheral blood offers a readily accessible source of cells for the investigation of lymphocyte function *in vitro*. Such studies have been carried out in many diseases including rheumatoid arthritis (Lockshin, Eisenhauer, Kohn, Block & Mushlin, 1975), multiple sclerosis (Jensen, 1968) and malignant states (Aisenberg, 1965). Prostaglandins, particularly of the E series, have been reported to inhibit both *in vitro* functions of lymphocytes (Smith, Steiner & Parker, 1971; Lichtenstein, Gillespie, Bourne & Henney, 1972; Berenbaum, Cope & Bundick, 1975; Gordon, Bray & Morley, 1976; Goodwin, Bankhurst & Messner, 1977) and *in vivo* responses mediated by lymphocytes (Quagliata, Lawrence & Phillips-Quagliata, 1973). We considered that the procedures commonly employed to isolate and culture lymphocyte-rich cell populations were likely to involve exposure of lymphocytes to prostaglandins from several sources. Prostaglandins have been shown to be generated during blood collection and centrifugation (Samuelsson, Granström, Gréen, Hamberg & Hammarström, 1975). Furthermore, prostaglandins are produced during mononuclear cell culture (Gordon *et al.*, 1976; Goodwin *et al.*, 1977) and accumulate in contrast to their rapid *in vivo* removal (Samuelsson *et al.*, 1975). Therefore, we examined the effects of agents which inhibit the formation of prostaglandins and other products of arachidonic acid metabolism present during cell preparation and/or the culture period, on phytohaemagglutinin (PHA)-induced [$^3$H]-thymidine incorporation by human peripheral blood mononuclear cell preparations. In addition, we examined the capacity of prostaglandin $E_2$ ($PGE_2$) prostacyclin ($PGI_2$) and its spontaneous decay product(s) to reverse the effect of such agents.

[1] Present address: Thrombosis Research Unit, The Rayne Institute, Kings College Hospital, 123 Cold Harbour Lane, London S.E.5.

## Methods

### *Preparation of mononuclear cells and serum*

Peripheral venous blood (30 ml aliquots) from apparently healthy volunteers who had received no medication within the previous 15 days was collected aseptically via 15 gauge siliconised needles into 50 ml plastic syringes containing 15 ml Dextraven 150 (Fisons), 700 units preservative-free heparin (Paines & Byrne) and, where appropriate, 90 μg indomethacin (Merck, Sharp & Dohme) or 450 μg eicosatetraynoic acid (Roche). After mixing by gentle inversion, the cells were allowed to sediment for 45 min at room temperature. The leukocyte-rich supernatant (12 ml aliquots) was layered onto Ficoll-Hypaque (8 ml 6.31 g Ficoll 400 (Pharmacia), 12.5 g Hypaque sodium (Winthrop) dissolved in 100 ml distilled water sp. gr. 1.077) in sterile, plastic, disposable universal con-

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tainers (Sterilin) and centrifuged at 450 *g* for 35 min at room temperature. The cells at the interface were removed, washed three times with HEPES (BDH)-buffered Eagles MEM (Wellcome) at room temperature, and resuspended at a concentration of $1.56 \times 10^6$ viable nucleated cells per ml in RPMI 1640 (Gibco), containing antibiotics (penicillin 100 u/ml and streptomycin 100 μg/ml, Glaxo) and supplemented with 10% autologous serum. Cell viability estimated by nigrosin exclusion was always >98% before cell culture. Such cell preparations contained 65 to 85% lymphocytes, 15 to 35% monocytes and <2% polymorphonuclear leukocytes with <2 erythrocytes per 100 leukocytes and 3 to 10 platelets per leukocyte, in agreement with results reported by Bøyum (1976). Where appropriate, the media and sera contained indomethacin (2 μg/ml) or eicosatetraynoic acid (10 μg/ml). Autologous sera were prepared from separate samples of blood collected at the same time in the presence or absence of indomethacin (2 μg/ml) or eicosatetraynoic acid (10 μg/ml).

*Cell cultures*

Aliquots (160 μl) of cell suspensions (containing $2.5 \times 10^5$ cells) were distributed in random manner into the wells of microtitre plates (round-bottomed, 250 μl capacity, Sterilin), excluding the outer-edge wells which contained 200 μl serum-free medium, and warmed to 37°C. Purified PHA (Wellcome) and prostaglandins or vehicle were added directly to the appropriate wells. Quintuplicate cultures were performed and treatments were randomly distributed throughout the plate. The plates were incubated at 37°C in an atmosphere of 10% $CO_2$ in humified air. The cultures were pulsed with 0.2 μCi [$^3$H]-thymidine (sp. act. 2 Ci/mmol, Radiochemical Centre, Amersham) after 48 h, and harvested onto glass-fibre filters (Whatman) 20 to 22 h later using a Mash II Harvester (Dynatech). The dried filters were placed in vials containing 5 ml scintillation fluid (PPO, 3 g/l and POPOP, 0.3 g/l in toluene) and counted in a liquid scintillation counter.

*Prostaglandins*

$PGE_2$ (Sigma) was stored at −20°C at a concentration of 1 mg/ml in absolute ethanol and was diluted in sterile RPMI medium before use. Prostacyclin (a gift from the Institute of Organic Chemistry, Syntex) was stored at −20°C in Tris buffer (0.2 M pH 8.6) and was diluted initially in sterile RPMI medium buffered with 10 mM Tris to pH 8.6. The final dilutions of $PGE_2$ and $PGI_2$ were made in equal volumes of RPMI medium (pH 7.4) containing the appropriate amount of PHA (5 μg/ml), and added immediately as a single aliquot (40 μl) to the cell cultures (160 μl).

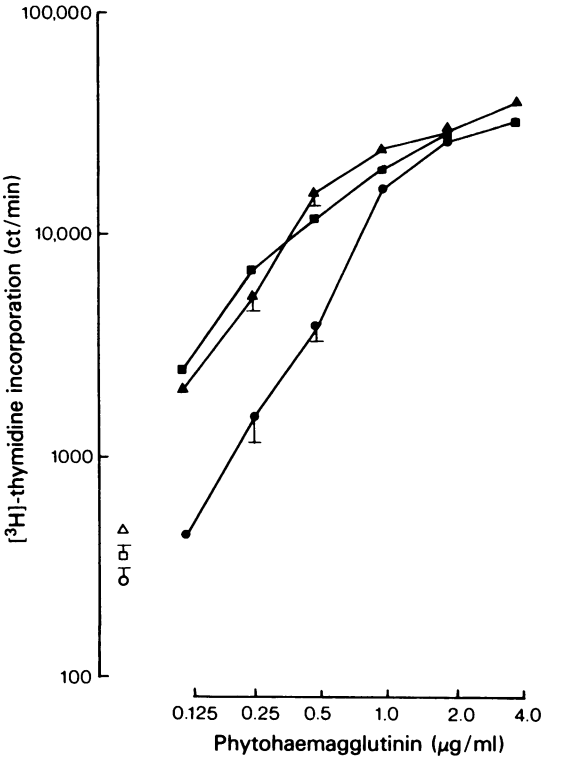


**Figure 1** [$^3$H]-thymidine incorporation by unstimulated (open symbols) and phytohaemagglutinin-stimulated (closed symbols) human peripheral blood mononuclear cells in the absence (○, ●) and presence of indomethacin, 2 μg/ml (△, ▲) or eicosatetraynoic acid, 10 μg/ml (□, ■). Each point is the mean of 5 replicate samples; vertical bars show s.e. mean.

## Results

*Effects of indomethacin and eicosatetraynoic acid on unstimulated and mitogen-induced [$^3$H]-thymidine incorporation*

Indomethacin (2 μg/ml) and eicosatetraynoic acid (10 μg/ml), present throughout blood collection, serum and cell preparation and *in vitro* culture, enhanced the dose-related PHA-induced [$^3$H]-thymidine incorporation by human peripheral blood mononuclear cells (Figure 1). The effects on unstimulated cells were negligible compared with the increase induced in mitogen-stimulated cells, i.e. the drugs were not mitogenic when used alone.

Figure 1 illustrates that 2 to 3 fold higher PHA concentrations were required to produce equivalent responses in untreated, compared with drug-treated cells from a single donor. Similar shifts (1.25 to 4

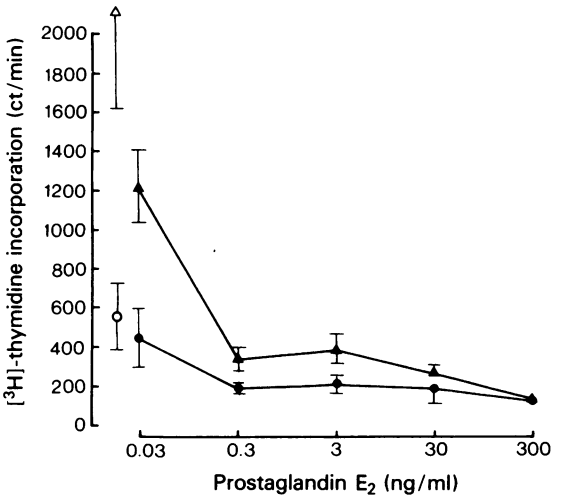


**Figure 2** Effect of prostaglandin $E_2$ ($PGE_2$, closed symbols) on phytohaemagglutinin (0.1 μg/ml) stimulated [$^3$H]-thymidine incorporation by human blood peripheral blood mononuclear cells in the presence (▲) or absence (●) of indomethacin (2 μg/ml). Control [$^3$H]-thymidine incorporation in the absence of added $PGE_2$ shown by corresponding open symbols. Each point is the mean of 5 replicate samples and the vertical bars indicate the s.e. mean.

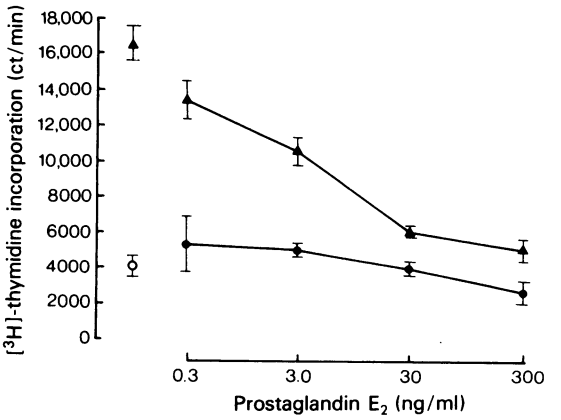


**Figure 3** Effect of prostaglandin $E_2$ ($PGE_2$, closed symbols) on phytohaemagglutinin (0.5 μg/ml) stimulated [$^3$H]-thymidine incorporation by human blood peripheral blood mononuclear cells in the presence (▲) or absence (●) of indomethacin (2 μg/ml). Control [$^3$H]-thymidine incorporation in the absence of added $PGE_2$ shown by corresponding open symbols. Each point is the mean of 5 replicate samples and the vertical bars indicate the s.e. mean.

fold) in the PHA dose-response curves were observed in indomethacin-treated cells from 8 other donors. However, although the maximum response obtained was similar in untreated and drug-treated cells, it was achieved at lower PHA concentrations and over a wider dose range in drug-treated cells. The responses to supramaximal (not shown) concentrations of PHA were less affected by indomethacin.

Similar results were obtained when indomethacin (2 μg/ml) was added to cultures of cells which had been collected and prepared in the absence of indomethacin. Indomethacin (2 μg/ml) also produced a comparable shift in the dose-response curve to purified protein derivative of tuberculin (Dept. Ag. Fish) when [$^3$H]-thymidine incorporation was measured between days 4 and 5 of culture in a single donor (data not shown).

*Effects of prostaglandins on mitogen-stimulated [$^3$H]-thymidine incorporation*

Cells from four donors were exposed to three PHA concentrations (0.1, 0.5 and 2.0 μg/ml) and the effects of exogenously added $PGE_2$ examined.

These results indicated that 'spontaneous' [$^3$H]-thymidine incorporation and that induced by threshold PHA concentrations, i.e. those where the response was only exposed by indomethacin treatment (cf 0.125 μg PHA/ml in Figure 1) were most susceptible to inhibition by exogenous $PGE_2$. Inhibition by low concentrations (0.03 ng/ml) of $PGE_2$ was detectable in these situations and [$^3$H]-thymidine incorporation could be reduced to about 100 ct/min (Figure 2). At moderate levels of mitogen-induced [$^3$H]-thymidine incorporation, as illustrated in Figure 3, inhibition by exogenous $PGE_2$ was detectable at lower concentrations in indomethacin-treated cells than in non-indomethacin-treated cells and maximum inhibition approximated to reversal of the enhancing ability of indomethacin. This implies that endogenous prostaglandin production is sufficient to produce the maximum inhibitory response to prostaglandin under such circumstances. At near optimal levels of [$^3$H]-thymidine incorporation, exogenously added $PGE_2$ (or $PGI_2$) had only a small inhibitory effect (10 to 20% maximum inhibition), whether or not the cells were indomethacin-treated (data not shown).

This pattern of results is quite compatible with the effects of indomethacin on the PHA dose-response curve (Figure 1). $PGE_2$ was tested in 4 other donors and similar results were obtained when sub-optimal concentrations of PHA were used.

Figure 4 shows that $PGI_2$ (0.03 to 0.3 ng/ml) inhibited in dose-related manner PHA-stimulated [$^3$H]-thymidine incorporation into both non-indomethacin-treated and indomethacin-treated cells. As seen with $PGE_2$, the maximum reduction in the response of indomethacin-treated cells to PHA corresponded to the difference in [$^3$H]-thymidine incorporation between indomethacin- and non-indometha-

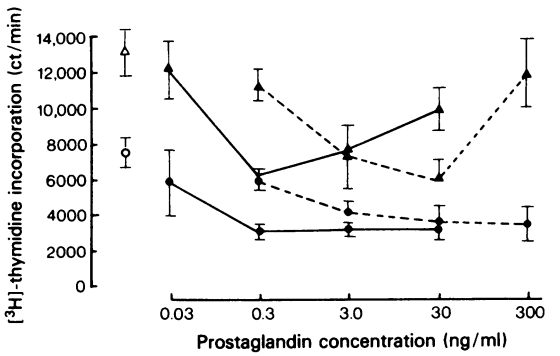


**Figure 4** Effect of prostacyclin ($PGI_2$, solid lines) and decayed $PGI_2$ (broken lines) on phytohaemagglutinin (0.5 μg/ml) stimulated [$^3$H]-thymidine incorporation by human peripheral blood mononuclear cells in the presence (▲) or absence (●) of indomethacin (2 μg/ml). Control [$^3$H]-thymidine incorporation in the absence of added $PGI_2$ shown by corresponding open symbols. Each point is the mean of 5 replicate samples and the vertical bars indicate the s.e. mean.

cin-treated cells. This effect was seen in cells from 4 other donors. At higher $PGI_2$ concentrations (3 to 30 ng/ml) there was no further reduction in [$^3$H]-thymidine incorporation by non-indomethacin-treated cells, whereas, in contrast, the inhibition was less in indomethacin-treated cells, i.e. the $PGI_2$ dose-response curve was bell-shaped in the indomethacin-treated cells. The results obtained with $PGI_2$ were paralleled by higher concentrations of 'decayed' $PGI_2$, which had been allowed to stand for 20 min at 37°C (Figure 4). We were unable to demonstrate inhibition by 6-keto-$PGF_{1\alpha}$ at comparable concentrations in three donors but cannot distinguish, on the basis of these experiments, whether the activity of 'decayed' $PGI_2$ was due to residual prostaglandin or to the formation of decay product(s) other than 6-keto-$PGF_{1\alpha}$ which retained a fraction of the activity of $PGI_2$.

$PGI_2$ rapidly lost its inhibitory effectiveness on preincubation with cells for more than 10 min prior to PHA administration, and was without effect on antigen-stimulated [$^3$H]-thymidine incorporation when added at the beginning of culture (data not shown).

## Discussion

The results show that indomethacin and eicosatetraynoic acid markedly enhanced mitogen-induced lymphocyte transformation (Figure 1). This is most readily interpreted in terms of the known inhibitory action of these agents on the fatty acid cyclo-oxygenase (Samuelsson *et al.*, 1975) which converts arachidonic acid to prostaglandin endoperoxides, derivatives of which include $PGE_2$ and $PGI_2$ (Johnson, Morton, Kinner, Gorman, McGuire, Sun, Whittaker, Bunting, Salmon, Moncada & Vane, 1976). Prostaglandins of the E series are known to inhibit *in vitro* lymphocyte responses (Smith *et al.*, 1971; Lichtenstein *et al.*, 1972; Berenbaum *et al.*, 1975; Gordon *et al.*, 1976; Goodwin *et al.*, 1977) (see also Figures 2 and 3). In addition, we have shown that $PGI_2$ reduced lymphocyte transformation (Figure 4). The indomethacin concentration (2 μg/ml) used in these experiments approximated to the peak drug concentration achieved in blood following administration of therapeutic doses in man (Holt & Hawkins, 1965), which are sufficient to inhibit by 77% to 98% whole body PGE production (Hamberg, 1972). More recently, while these experiments were in progress, it has been shown independently that comparable concentrations of indomethacin (1 μg/ml) present during the period of cell culture enhanced maximal PHA mitogen-stimulated [$^3$H]-thymidine incorporation (Goodwin *et al.*, 1977) although less dramatically than in our experiments.

The endogenous levels of primary prostaglandins in peripheral plasma are low; for example, that of $PGE_2$ is of the order of a few picograms per ml (Samuelsson *et al.*, 1975). The concentration of $PGI_2$ is unknown. However, prostaglandins may be rapidly biosynthesized by cellular elements during blood collection, and more especially during blood clotting (Silver, Smith, Ingerman & Kocsis, 1972), reaching levels (0.3 to 30 ng/ml) which are sufficient to inhibit mitogen stimulation of lymphocyte responses (Figure 3). Thus the use of serum to supplement tissue culture medium is likely to indtroduce variable amounts of prostaglandins into the experimental system (commercially available sera have been reported to contain several hundred picograms $PGE_2$ per millilitre) (Ritzi & Stylos, 1974).

The present investigation has shown that indomethacin-treated mononuclear cells are more susceptible (approximately 100 fold) than untreated cells to inhibition by $PGE_2$ (Figure 3). This suggests that conventional methods of cell preparation may expose lymphocytes to various amounts of endogenously produced prostaglandins which would affect cell reactivity to PHA and to $PGE_2$. This may account for the differences in inhibitory potency of $PGE_2$ observed between this and other studies (Smith *et al.*, 1971; Berenbaum *et al.*, 1975; Goodwin *et al.*, 1977).

$PGI_2$, at low concentrations, inhibited lymphocyte transformation in normal and indomethacin-treated cells (Figure 4). However, inhibition by higher concentrations of prostacyclin may be dependent upon intact fatty acid cyclo-oxygenase, since indomethacin-treated cells exhibited a bell-shaped response curve to prostacyclin.

Another possibility is that high concentrations of $PGI_2$ elevate intracellular levels of cyclic nucleotides other than adenosine monophosphate, which have opposing actions on cell proliferation (Strom, Lundin & Carpenter, 1977), and which are possibly enhanced by indomethacin.

Studies in experimental animals and man have implicated a negative feedback role for prostaglandins (not wholly accounted for by $PGE_2$) produced by cells of the monocyte-macrophage series, on lymphocytes during *in vitro* culture (Gordon *et al.*, 1976; Goodwin *et al.*, 1977). Evidence has been presented that excessive prostaglandin production could contribute to lymphocyte anergy in malignant disease (Pelus & Strausser, 1976; Goodwin, Messner, Bankhurst, Peake, Saiki & Williams, 1977). In addition our results indicate that $PGI_2$ should now be considered to have a role as a modulator of lymphocyte function.

Prostacyclin production by macrophages implied by the detection of 6-keto-$PGF_{1\alpha}$ (Chang, Muroto, Matsuo & Tsurufugi, 1976; Humes, Bonney, Pelus, Dahlgren, Sadowski, Kuehl & Davies, 1977) may now be considered to supplement $PGE_2$-mediated lymphocyte suppression previously described. Furthermore, uterine (Fenwick, Jones, Naylor, Poyser & Wilson, 1977) and placental (Myatt & Elder, 1977) production of prostacyclin could contribute to lymphocyte anergy associated with the later stages of pregnancy. This latter phenomenon has been attributed to the interaction of polyamines in foetal tissue with polyamine oxidase (Byrd, Jacobs & Amoss, 1977; Allen, Smith, Curry & Gaugas, 1977; Gaugas & Curzen, 1978) which is elevated in serum of pregnant women. Polyamine-induced suppression of lymphocytes is reversible by indomethacin (Jacobs & Byrd, 1977) indicating mediation by prostaglandins.

Prostacyclin production by endothelial cells (Harker, Joy, Wall, Quadracci & Striker, 1977) could be considered to have a physiological role in limiting intravascular activation of lymphocytes, as has been suggested previously with respect to platelets (Moncada, Herman, Higgs & Vane, 1977; Westwick, 1977). The potency of prostacyclin would be adequate for such a role on circulating lymphocytes, while the reversibility of action would allow the return of extravascular lymphocytes to their full potential.

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