*Br. J. Pharmac.* (1980), **68**, 199–205

# FAILURE OF ANTI-INFLAMMATORY STEROIDS TO INHIBIT PROSTAGLANDIN PRODUCTION BY RAT POLYMORPHONUCLEAR LEUCOCYTES

F. DRAY, ELAINE McCALL[1] & L.J.F. YOULTEN*

U.R.I.A., Institut Pasteur, 28, rue du Docteur Roux, 75015 Paris, France &
*Department of Medicine, Guy's Hospital Medical School, London SE1 9RT

**1** Like rabbit polymorphonuclear (PMN) leucocytes, rat peritoneal glycogen-induced PMN leucocytes produced much greater amounts of prostaglandin when incubated with killed bacteria than in the absence of phagocytosable material.

**2** Rat PMN leucocytes produced mainly prostaglandin $E_2$ ($PGE_2$), in amounts up to 17 ng/$10^6$ cells in 90 min incubation, some 25 times the amount produced by resting cells.

**3** Indomethacin and meclofenamic acid inhibited prostaglandin production by resting and phagocytosing cells, the $IC_{50}$ being of the order of $10^{-6}$ to $10^{-7}$ M for both drugs.

**4** Hydrocortisone and dexamethasone at concentrations up to $10^{-4}$ M did not cause significant dose-related inhibition of prostaglandin production in this system.

**5** It is suggested that the phagocytosing PMN leucocyte is insensitive to the action of anti-inflammatory steroids with respect to prostaglandin production.

## Introduction

Prostaglandins may be mediators of the inflammatory response (Ferreira & Vane, 1974). They have been detected in many inflammatory lesions including inflamed joints (Moncada, Ferreira & Vane, 1974; Higgs, Vane, Hart & Wojtulewski, 1974; Blackham, Farmer, Radziwonik & Westwick, 1974), skin (Greaves & Søndergaard, 1970) and eyes (Eakins, Whitelocke, Perkins, Bennett & Unger, 1972). Non-steroid anti-inflammatory drugs inhibit prostaglandin synthesis in a wide variety of preparations (Flower, 1974) and this may be the mechanism of their anti-inflammatory action. More recently it has been proposed that the action of steroid anti-inflammatory drugs may also involve interference with prostaglandin production (Gryglewski, Panczenko, Korbut, Grodzinska & Ocetkiewicz, 1975; Blackwell, Flower, Nijkamp & Vane, 1978).

One possible source of the prostaglandins in acute inflammation is the polymorphonuclear (PMN) leucocyte. PMN leucocytes have been shown to release prostaglandins during phagocytosis (Higgs, McCall & Youlten, 1975; Zurier, 1976) and in some inflammatory exudates there is a correlation between the numbers of PMN leucocytes present and the prostaglandin levels (Anderson, Brocklehurst & Willis, 1971; Eakins *et al.*, 1972; Higgs *et al.*, 1974). We have previously measured prostaglandin production by phagocytosing rabbit PMN leucocytes and its inhibition by non-steroid anti-inflammatory drugs (Higgs *et al.*, 1975). We have now studied prostaglandin production by phagocytosing rat PMN leucocytes and the effect on this of both steroid and non-steroid anti-inflammatory drugs.

## Methods

Male Wistar rats (200 to 250 g) were lightly anaesthetized with ether and given intraperitoneal injections of 20 ml 0.1% w/v glycogen in 0.9% w/v NaCl solution (saline). Four hours later the animals were killed with ether and the peritoneal cavity was opened and washed out with 10 ml Hanks basic salt solution containing 50 u/ml heparin. The resulting cell suspension was aspirated into plastic tubes.

Viability, total and differential cell counts were measured on small samples and the rest of the cell suspension was centrifuged at 60 *g* for 10 min. The supernatant was discarded and the cell pellet resuspended to give a concentration of $3 \times 10^6$ cells/ml, in Hanks solution containing 1 mg/ml glucose and 100

[1] Present address: Academic Unit of Immunology, The Rayne Institute, St Thomas's Hospital, London SE1 7EH.

0007-1188/80/020199-07 $01.00

μg/ml bovine serum albumin. The resulting 3 to 5 ml suspension was placed in 5 plastic tubes, to which were added either drugs dissolved in the appropriate vehicle or vehicle alone. The cells were incubated at 37°C for 2 h and then centrifuged at 60 *g* for 10 min. The supernatant was discarded and the cells resuspended in the same volume of fresh medium. The same concentrations of drugs were then added. The suspensions were divided into two portions, to one of which was added killed *Bordetella pertussis* to a final concentration of 1000 bacteria/cell. The cell suspensions were incubated at 37°C and samples were removed at various times. After centrifugation at 60 *g* for 10 min the resulting supernatants were stored at −20°C until assay.

*Radioimmunoassay*

Assay of prostaglandins was carried out by means of a sensitive and specific radioimmunoassay (Dray, Charbonnel & Maclouf, 1975). To tubes containing 0.1 ml standard prostaglandin solution or dilution of the sample were added 8000 d/min [$^3$H]-prostaglandin in 0.1 ml and 0.1 ml antiserum diluted to give approximately 40% binding in the absence of added standard prostaglandin. After incubation at 4°C for 2 h, the free and bound prostaglandin were separated by dextran charcoal. The resulting supernatant containing the antibody-bound radioactivity was added to 10 ml scintillating fluid and counted in an Intertechnique automatic beta-counter.

*Drugs*

Hydrocortisone and dexamethasone were dissolved in absolute ethanol, indomethacin was dissolved in 5% (w/v) $NaHCO_3$/water, and meclofenamic acid in Tris HCl buffer 0.1 M pH 8.4. Dilutions were made in the same vehicle, and drugs at one hundred times the final required concentration were added to the cells in a volume of 10 μl/ml cell suspension.

*Materials*

Hanks TC medium was obtained from Institut Pasteur, and chemically killed *Bordetella pertussis* (Per/Vac) from Burroughs Wellcome. Shellfish (type II) or rabbit liver (type III) glycogen, dexamethasone and hydrocortisone were obtained from Sigma. [5,6 (n) $^3$H]-prostaglandins $E_1$, $E_2$, and $F_{2\alpha}$ were obtained from the Radiochemical Centre, Amersham.

Indomethacin was a gift from Merck, Sharpe and Dohme, and meclofenamic acid from Parke Davis Co. Prostaglandin $E_2$ was a gift from Dr J. E. Pike (Upjohn).

## Results

*Characterization of cell suspensions*

The volume of fluid aspirated was 14.0 ± 3.2 ml (mean ± s.e. mean) (20 experiments) and the total cell count 91.1 ± 32.7 × $10^6$/ml. Viability was always greater than 99%, as measured by exclusion of trypan blue, at the beginning of the experiment and never fell below 95% by the end. Differential counts of stained films showed that more than 80% of the cells were PMN leucocytes, the remainder being principally macrophages.

*Validation of the assay*

Antiserum to $PGF_{2\alpha}$ cross-reacted with $PGF_{1\alpha}$ 30%, and that to $PGE_1$ cross-reacted with $PGE_2$ 20%.

The anti-$PGE_2$ serum cross-reacted 6% with $PGE_1$ and less than 0.1% with $PGF_{2\alpha}$, $PGF_{1\alpha}$, $PGD_2$ or 6-keto $PGF_{1\alpha}$. The sensitivity was such that 2 to 3 pg could be measured. The binding parameters of these antisera have been described previously (Dray *et al.*, 1975; Gerozissis & Dray, 1977). Preliminary experiments demonstrated that direct assay of unextracted supernatants gave results comparable with those obtained after extraction with organic solvents and silicic acid chromatography. Neither bacteria nor drugs at the concentrations used in these experiments interfered significantly with the radioimmunoassay of $PGE_2$.

The recovery of standard prostaglandins added to the medium immediately before assay was better than 95%.

The inter- and intra-assay precision of the method were also measured. A known amount of prostaglandin was measured in 5 separate assays, with a coefficient of variation of 11% (inter-assay precision) and in 9 duplicates in a single assay, with a coefficient of variation of 5.3% (intra-assay precision).

*Prostaglandin production by rat polymorphonuclear leucocytes*

*Type* The principal prostaglandin produced by rat PMN leucocytes was assayed as $PGE_2$, and in all experiments $PGF_{2\alpha}$ and $PGE_1$ levels were never more than 10% of the $PGE_2$ levels. Incubation of cells with bacteria (which contained no prostaglandins nor substrates for their synthesis) increased the production of prostaglandins. Prostaglandin $E_2$ production, for example, rose to as much as 17 ng/$10^6$ cells in 90 min. After 90 min incubation, supernatants from cells incubated with bacteria contained 25 times as much $PGE_2$ as supernatants from non-phagocytosing cells (Figure 1).

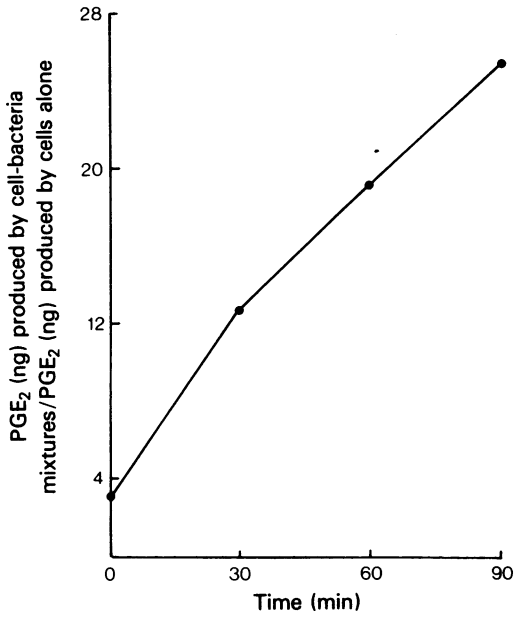


**Figure 1** The ratio of phagocytosing/non-phagocytosing prostaglandin $E_2$ production by rat polymorphonuclear leucocytes.

Cell-free Hanks medium was assayed in the conditions described above. In 5 experiments, levels of $PGE_2$ ranged from 2 to 4 pg/ml and $PGF_{2\alpha}$ from 20 to 25 pg/ml. Levels of $PGE_1$ were undetectable. Stability of prostaglandins during the incubation was measured by adding [$^3$H]-PGE (0.07 μCi) to 20 ml cell-free culture medium and incubating at 37°C. After 30 min incubation, the medium was submitted to an extraction procedure and a purification step in which prostaglandin metabolites are separated from pure prostaglandins. Degradation (expressed as the ratio of the radioactivity in the metabolite fraction to the total radioactivity in all fractions) was of the order of 4%/30 min.

*Effect of non-steroid anti-inflammatory drugs* Cells were incubated with vehicle only or with indomethacin or meclofenamic acid at $10^{-8}$ to $10^{-5}$ M. Inhibition of prostaglandin production was observed both in phagocytosing and in resting cells. Preliminary experiments showed that $PGE_2$ and $PGF_{2\alpha}$ production were affected similarly. The inhibition caused by indomethacin was dose-dependent (Figure 2) and equally effective if the cells were not pre-incubated with the drug for 2 h before addition of bacteria. Viability of the cells was unchanged. In three experiments the $IC_{50}$ was $1.8 \times 10^{-7}$ M, $4 \times 10^{-7}$ M and $9.1 \times 10^{-7}$ M.

Meclofenamic acid at concentrations between $10^{-8}$ and $10^{-5}$ M also inhibited prostaglandin production

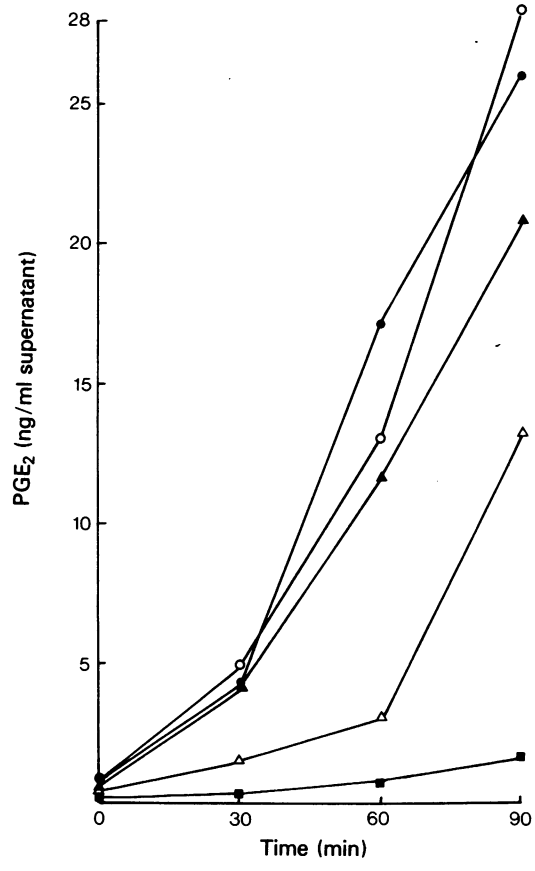


**Figure 2** The effect of indomethacin (final concentration $10^{-8}$ M to $10^{-5}$ M) on prostaglandin production by phagocytosing rat polymorphonuclear leucocytes. Control (●); indomethacin $10^{-8}$ M (○); $10^{-7}$ M (▲); $10^{-6}$ M (△); $10^{-5}$ M (■).

in a dose-dependent manner. One such experiment is shown in Figure 3. Results were similar for $PGE_2$ and $PGF_{2\alpha}$. Viability of the cells was not altered. In three experiments the $IC_{50}$ was $3.5 \times 10^{-7}$ M, $7.8 \times 10^{-7}$ M and $1.9 \times 10^{-6}$ M.

*Effect of anti-inflammatory steroids* Hydrocortisone and dexamethasone were dissolved in 90% ethanol and added to the cell suspensions to give final concentrations of $10^{-10}$ to $10^{-4}$ M. Ethanol added in these amounts (10 μl/ml cell suspension) did not alter prostaglandin production by either the phagocytosing or resting cells. Prostaglandin production by phagocytosing cells in the presence of different concentrations of these drugs is shown in Figures 4 and 5 and in Table 1. The production of prostaglandins by the PMN leucocytes was not inhibited with increasing dose or time and in some cases was even increased.

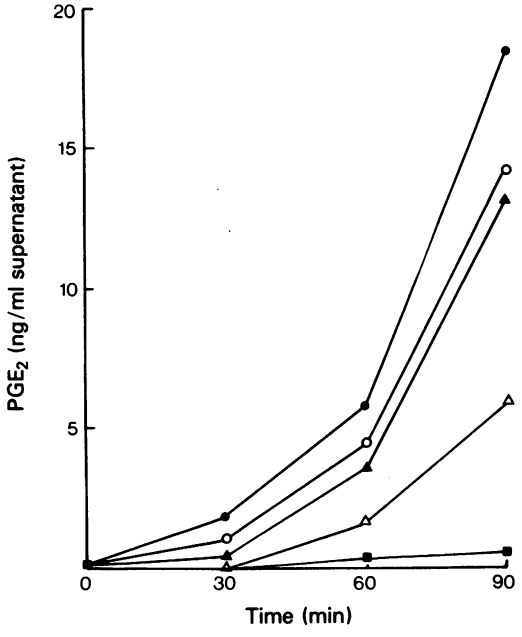


**Figure 3** The effect of meclofenamic acid (final concentration $10^{-8}$ M to $10^{-5}$ M) on prostaglandin production by phagocytosing rat polymorphonuclear leucocytes. Control (●); meclofenamic acid $10^{-8}$ M (○); $10^{-7}$ M (▲); $10^{-6}$ M (△); $10^{-5}$ M (■).

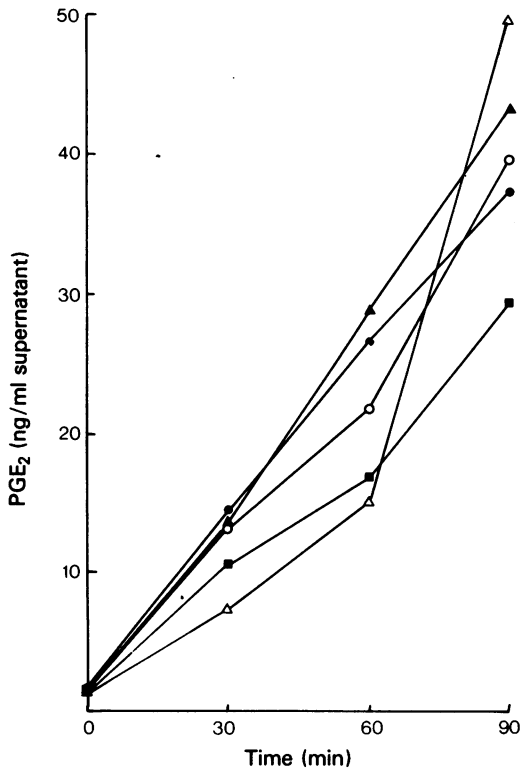


**Figure 4** The effect of dexamethasone (final concentration $10^{-10}$ M to $10^{-4}$ M) on prostaglandin production by phagocytosing rat polymorphonuclear leucocytes. Control (●); dexamethasone $10^{-10}$ M (○); $10^{-8}$ M (▲); $10^{-6}$ M (△); $10^{-4}$ M (■).

Prostaglandin production by resting cells, and viability of both resting and stimulated cells was unaltered. Similar results were seen for production of $PGF_{2\alpha}$.

## Discussion

*Production of prostaglandins*

Rat PMN leucocytes produce prostaglandins during incubation with killed bacteria. Up to 17 ng $PGE_2/10^6$ cells is produced in the first 90 min. This is considerably more than the amounts produced by rabbit PMN leucocytes: in 5 experiments phagocytosing rabbit PMN leucocytes incubated in modified Hanks medium at 37°C for 2 h produced between 0.2 and 1.7 ng PGE-like material/$10^6$ cells. Furthermore, rabbit PMN leucocytes produced an appreciable quantity of $PGE_1$: AII chromatography carried out

**Table 1** Effect on prostaglandin $E_2$ production by phagocytosing polymorphonuclear (PMN) leucocytes of dexamethasone and hydrocortisone

| Concentration (M) | $10^{-10}$ | $10^{-8}$ | $10^{-6}$ | $10^{-4}$ |
|---|---|---|---|---|
| Dexamethasone | 76.5 (63.7 – 83.2) | 86.9 (80.4 – 99.1) | 81.1 (61.0 – 93.3) | 91.9 (35.4 – 135.0) |
| Hydrocortisone | 71.3 (31.0 – 92.7) | 85.0 (43.2 – 101.1) | 76.9 (32.7 – 98.9) | 68.6 (38.8 – 107.5) |

Prostaglandin levels after 90 min incubation as percentage of control (no steroid). Values are mean (and range) of 5 experiments.

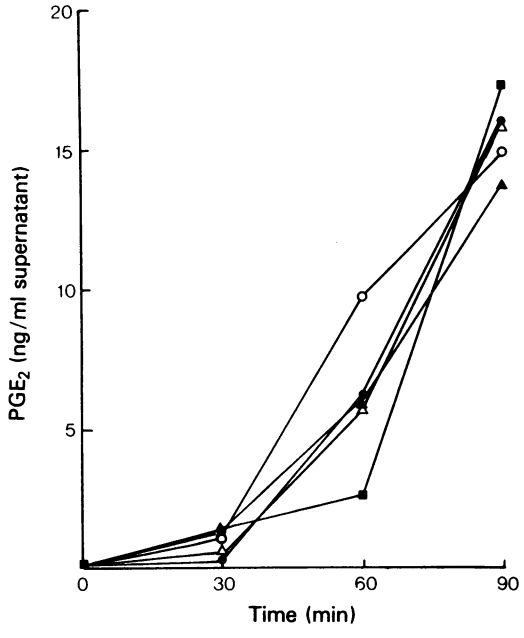


**Figure 5** The effect of hydrocortisone (final concentration $10^{-10}$ M to $10^{-4}$ M) on prostaglandin production by phagocytosing rat polymorphonuclear leucocytes. Control (●); hydrocortisone $10^{-10}$ M (○); $10^{-8}$ M (▲); $10^{-6}$ M (△); $10^{-4}$ M (■).

on 3 samples showed that 39 to 80% of the PGE-like activity corresponded to authentic $PGE_1$, both in its $R_F$ value and in its biological activity (Higgs *et al.*, 1975). This variation in the type of prostaglandin produced may represent a species difference. In rabbit inflammatory exudates in which infiltration of PMN leucocytes occurs and in which these cells may be a major source of prostaglandins, there are considerable quantities of $PGE_1$ (Eakins *et al.*, 1972; Blackham *et al.*, 1974), whereas the principal stable prostaglandins in rat inflammatory exudates are $PGE_2$ and $PGF_{2\alpha}$ (Willis, 1969).

The difference in the amounts of prostaglandin produced is unlikely to be due to the type of cell present. Macrophages, which are present in relatively small numbers (up to 20%) in the rat peritoneal cell suspensions used, do not produce prostaglandins in such large amounts in such a short time (90 min) in the absence of serum (McCall, unpublished observations).

*Effects of inhibitors*

We have previously described the ability of indomethacin to inhibit prostaglandin synthesis by rabbit PMN leucocytes (Higgs *et al.*, 1975). This could be relevant to the anti-inflammatory effect of this drug, resulting as it does in a fall in prostaglandin levels and perhaps also in a reduction of PMN migration. In the present experiments both indomethacin and meclofenamic acid inhibited prostaglandin release by rat PMN leucocytes in a dose-dependent manner, with $IC_{50}$s in the expected range.

The anti-inflammatory steroids have been widely used in treating skin, lung, gastrointestinal, joint and other inflammatory states. Recently the effects of steroids on prostaglandin metabolism have been studied. High concentrations of anti-inflammatory steroids inhibited prostaglandin synthetase in skin homogenates (Greaves & McDonald-Gibson, 1972) while other authors observed no inhibition at moderate concentrations (Flower, Gryglewski, Herbacynska-Cedro & Vane, 1972). However, in several whole cell systems, steroids caused a reduction of prostaglandin production (Gryglewski *et al.*, 1975; Kantrowitz, Robinson, McGuire & Levine, 1976). Gryglewski *et al.* (1975) proposed that steroids reduced the availability of substrate for prostaglandin synthesis, and Hong & Levine (1976) observed that steroids could inhibit arachidonic acid release from phospholipids in serum-stimulated transformed mouse fibroblasts and suggested that this might be the mechanism of their anti-inflammatory action. Blackwell *et al.* (1978) found that steroids reduced phospholipase $A_2$ activation in guinea-pig lungs both under basal conditions and when stimulated by various mechanical, chemical and immunological factors.

In the present experiments, steroids used in the same concentrations as used by Hong & Levine (1976) failed to inhibit prostaglandin production by rat PMN leucocytes. In some experiments there was even an apparent increase in the amount of prostaglandin produced. Our early experiments gave similar results, but in the present study all cells were pre-incubated with drugs for 2 h before addition of bacteria, since it had been reported that such a period of pre-incubation was necessary to demonstrate high levels of inhibition (Hong & Levine, 1976). In one series of experiments, rats were injected three times intraperitoneally with 1 mg/kg betamethasone sodium phosphate in the 24 h before induction of a sterile peritonitis by glycogen in the usual way. Total cell numbers in the exudate were slightly, though not significantly, reduced (mean, $54.9 \times 10^6$ in 5 experiments) and prostaglandin production by these cells was not significantly different from that of untreated cells.

It is not yet clear why there is an apparent difference in sensitivity to anti-inflammatory steroids between polymorphonuclear leucocytes and other cell types (e.g. synovial cells, fibroblasts and lung). Phospholipase $A_2$ activation probably occurs during phagocytosis, since phospholipase $A_2$ activity is associ-

ated with rabbit PMN leucocyte granules (Franson, Patriarca & Elsbach, 1974) and phospholipases are involved in the breakdown of phospholipids of ingested micro-organisms (Patriarca, Beckerdite, Pettis & Elsbach, 1972). The activation of phospholipase $A_2$ in phagocytosing PMN leucocytes may be an example of a 'steroid insensitive' mechanism, as proposed by Blackwell *et al.* (1978). This hypothesis was advanced to explain the fact that bradykinin-induced activation of phospholipase $A_2$ (unlike antigen, mechanical, histamine or RCS-RF-induced activation) was not inhibited by anti-inflammatory steroids. Chandrabose, Lapetina, Schmitges, Siegal & Cuatrecasas (1978) have reported that fibroblast cyclo-oxygenase was stimulated by steroids. The lack of effect of steroids on resting cell production of prostaglandins argues against a similar effect in PMN leucocytes, but a combination of inhibition of phospholipase $A_2$ and stimulation of cyclo-oxygenase could produce results such as those reported here. Phospholipase and cyclo-oxygenase activities in these cells are currently being investigated in our laboratory.

Levels of prostaglandin were not reduced in joint fluid in rheumatoid arthritis patients treated only with steroids (Higgs *et al.*, 1974). It is possible that this indicates PMN involvement. Either the PMN leucocytes themselves are a source of the prostaglandins or else the action of their lysosomal enzymes may produce prostaglandins from other sources (Anderson *et al.*, 1971). Both of these mechanisms would continue to produce prostaglandins in the presence of steroids.

Hydrocortisone and dexamethasone at concentrations up to $10^{-3}$ M have previously been shown to be inactive as stabilizers of human PMN leucocyte lysosomal membranes (Persellin & Ku, 1974) although hydrocortisone has previously been shown to stabilize liver lysosomes (Weissmann & Thomas, 1963). Thus the PMN leucocytes may represent a cell type generally insensitive to steroid action.

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*(Received November 2, 1978.*
*Revised May 7, 1979.)*