A CHEMOTACTIC ROLE FOR PROSTA-GLANDINS RELEASED FROM POLYMORPHO-NUCLEAR LEUCOCYTES DURING PHAGOCYTOSIS

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1   Prostaglandin E_1 is chemotactic at concentrations down to 10 ng/ml for rabbit polymorphonuclear (PMN) leucocytes. Prostaglandins E_2 and $F_{2\alpha}$ have little or no chemotactic effect at concentrations up to 10 μg/ml.

2   Washed PMN leucocytes produce a chemotactic agent during phagocytosis, but not in the presence of indomethacin (28 μM).

3   Phagocytosing PMN leucocytes produce up to ten times as much prostaglandin as do resting cells. Some of this is prostaglandin E_1 as judged by thin layer chromatography and differential bioassay. This prostaglandin production by PMN leucocytes is abolished by indomethacin (28 μM).

4   Ultrasonicated suspensions of PMN leucocytes produce prostaglandin from arachidonic acid. This synthesis is inhibited by indomethacin.

5   Homogenates of PMN leucocytes which have been pre-incubated with bacteria for 30 min show more prostaglandin synthetase activity than homogenates from PMN leucocytes which have not been exposed to bacteria.

6   It is concluded that in some forms of inflammation, prostaglandin E_1 may play a controlling role in cellular migration.

7   PMN leucocytes may contribute to the generation of prostaglandins found in some inflammatory lesions.

Introduction

Some inflammatory exudates contain prosta-glandins in concentrations high enough to mediate certain features, such as oedema and erythema, of the inflammatory response (Willis, 1969; Crunk-horn & Willis, 1971). Several non-steroid anti-inflammatory drugs at therapeutic concentrations inhibit prostaglandin synthesis in various systems (Vane, 1971; Smith & Willis, 1971; Ferreira, Moncada & Vane, 1971). Thus, prostaglandins may make a significant contribution to certain forms of inflammation. Although many different tissues release prostaglandins when suitably stimu-lated, there is evidence that in at least one form of experimental inflammation, immunogenic uveitis in rabbits (Eakins, Whitelocke, Perkins, Bennett & Unger, 1972) the source of the prostaglandin is the polymorphonuclear (PMN) leucocytes, cells in which we have demonstrated the capacity to produce prostaglandins during phagocytosis (Higgs & Youlten, 1972).

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Prostaglandin E_1 is chemotactic for rabbit PMN leucocytes (Kaley & Weiner, 1971). The present investigation explores the possibility that prosta-glandins released from PMN leucocytes might have a controlling role in attracting more cells to sites where phagocytosis is occurring. We have also investigated the amounts and types of prosta-glandin released from PMN leucocytes *in vitro* to see if they are likely to make an important contribution *in vivo*. Some of these results have been communicated to the British Pharmacological Society (Higgs & Youlten, 1972) and to the Physiological Society (McCall & Youlten, 1973).

Methods

Harvesting of polymorphonuclear leucocytes

PMN leucocytes were obtained by the method of Hirsch & Church (1960). Six adult New Zealand White rabbits were each used in rotation once every 7-10 days. A sterile peritonitis was induced

by the intra-peritoneal administration of 200 ml 0.9% w/v sterile NaCl solution (saline) containing 0.1% w/v glycogen. Four hours later the peritoneal cavity was washed out through a plastic cannula with 100 ml Hanks basic tissue culture medium, and the contents collected into siliconized glass tubes. A total cell count was made on one sample (0.1 ml) and another (0.1 ml) stained for a differential cell count. The remainder was centrifuged at 600 rev/min ($52 \times g$) for 10 min, the supernatant was poured off and the cells resuspended to the required concentration in Hanks solution enriched with glucose (0.56 mM) and bovine serum albumin (100 μg/ml). Total cell counts varied between 1×10^6/ml and 15×10^6/ml; by differential count, over 98% of the cells were PMN leucocytes. On the few occasions when there was erythrocyte contamination or the viability (measured by the exclusion of trypan blue dye, 10 mg/ml) was below 95%, the cells were discarded.

Chemotaxis

Chemotactic activity was measured with a perspex chamber separated by a Millipore filter (SCWPO 1300 pore size 8 μm) into two compartments (Boyden, 1962). Test solutions or suspensions were placed in the lower compartment, and cell suspensions in the upper one. After incubation at 37°C for 3 h, the filter was stained and its lower surface examined for stained cells, the number of cells being counted in each of nine randomly selected high power fields. This number is taken as an index of chemotactic attraction by the contents of the lower compartment for the cells in the upper compartment. In control experiments, the suspected chemotactic substance was added in equal concentrations to both compartments.

Prostaglandin production by phagocytosing cells

PMN leucocytes were resuspended in enriched Hanks medium to a concentration of 3.5-8.0 x 10^6 cells/ml. A portion (20 ml) of this suspension was put in each of two wide-necked tubes and to one killed bacteria (100/cell) were added. The tubes were lightly stoppered and incubated in a water bath at 37°C for up to 3 hours. The cells were then centrifuged ($52 \times g$; 10 min) and the supernatants collected. Each sample was acidified to pH 3 with 0.1 N HCl and extracted twice with equal volumes of ethyl acetate. The two extracts were pooled and evaporated to dryness under reduced pressure. For bioassay of prostaglandins, the residue was dissolved in 1 ml Krebs bicarbonate solution and a portion of this injected over a rat stomach fundus

strip (Vane, 1957), superfused at 5 ml/min with Krebs bicarbonate solution at 37°C containing a mixture of antagonists to prevent the action of acetylcholine, 5-hydroxytryptamine, histamine or the catecholamines (Gilmore, Vane & Wyllie, 1968). For thin-layer chromatography, the residue was dissolved in 5 ml ethanol/water (2:1, v/v) and partitioned four times against petroleum ether (b.p. 40-60°C). The aqueous ethanol phase was evaporated under reduced pressure, the residue redissolved in 0.25 ml methanol and applied to thin layer silica plates along with prostaglandin standards. The plates were developed in the AI or AII system (Gréen & Samuelsson, 1964) and divided into strips of 1 cm, which were scraped into tubes and eluted with 1 ml Krebs solution. After centrifugation, the resulting supernatants were bioassayed against prostaglandin standards on rat stomach fundus strip, chick rectum and rat colon. Small quantities (0.36 ng) of [5,6-(n)^{3}H]-prostaglandin E$_1$ (Radiochemical Centre, Amersham) were added to the samples before extraction and 0.1 ml aliquots of the final eluants were counted in a Packard Tricarb Liquid Scintillation system.

Prostaglandin synthesis by cell sonicates

PMN leucocytes were resuspended in 100 mM cold phosphate buffer (pH 7.4) at a concentration of 50-100 x 10^6 cells/ml. They were ultrasonicated at 20,000 Hz for 2 min and the resulting homogenate was used as the enzyme preparation. Prostaglandin synthetase activity was measured by the method of Flower, Gryglewski, Herbaczynska-Cedro & Vane (1972). The reaction mixture contained arachidonic acid (20 μg), reduced glutathione (100 μg), hydroquinone (10 μg) and cell homogenate (0.5 ml), and was made up to 2.0 ml with phosphate buffer. The reaction was started by adding the enzyme to the other components and samples were taken immediately after this addition (zero-time) and after 30 min incubation at 37°C, and the reaction was stopped by boiling for 45 seconds.

The amount of prostaglandin-like material produced by the enzyme system was assayed in terms of prostaglandin E$_2$ on a rat stomach strip superfused as previously described, with the addition of indomethacin (1 μg/ml) to prevent intrinsic prostaglandin formation by the tissue (Eckenfels & Vane, 1972).

In other experiments, PMN leucocytes were resuspended to a concentration of 5 x10^6 cells/ml in modified Hanks solution and incubated at 37°C for 30 min, either alone or in the presence of *Bordetella pertussis* in a ratio of 1000 bacteria per cell. The suspensions were then centrifuged

(52 x g) for 10 min, the supernatants poured off and the cells resuspended in a small volume of cold phosphate buffer and ultrasonicated. The resulting homogenates were then tested as above for their prostaglandin synthesizing activity. In each sample, the total protein was measured by the biuret method.

Materials

Substances used were Hanks TC medium (Difco), shellfish (type II) or rabbit liver (type III) glycogen (Sigma), chemically killed *Bordetella pertussis* (Per/Vac, Burroughs Wellcome), indomethacin (Merck Sharp & Dohme), prostaglandins E_1, E_2 and $F_{2\alpha}$ (Upjohn) and $[5,6(n)^3 H]$-prostaglandin E_1 (Radiochemical Centre, Amersham).

Results

Chemotactic activity of prostaglandins

When the lower compartment of the Boyden chamber contained sterile saline, after 3 h incubation, 20-75 cells were counted in 9 high power fields on the lower surface of the Millipore filter. The cell viability, tested by the exclusion of trypan blue dye at the end of each incubation was always greater than 90%.

Prostaglandin E_1 (1 μg/ml) was always chemotactic compared with the saline controls (Table 1). The mean increase was 3.6-fold with a range of 2.2-5.8. The differences between the means were significant ($P < 0.02$ by Student's paired t test).

Table 2 shows the chemotactic response induced by different concentrations of prostaglandins E_1, E_2 and $F_{2\alpha}$ from 10 ng/ml to 10 μg/ml. Prostaglandin E_1 appeared to have chemotactic activity at all concentrations tested. The effects of prostaglandins E_2 and $F_{2\alpha}$ were comparatively small. At 1 μg/ml, for example, prostaglandins E_2 and $F_{2\alpha}$ caused mean increases of only 1.1 and 1.2 times the control values. In five experiments, no increase in cell migration above control was seen when the upper compartment contained the same concentration of prostaglandin E_1 as the lower.

Chemotactic effect of phagocytosing cells

The chemotactic effect of phagocytosing cells was measured in 7 experiments. Dead bacteria were added to a cell suspension in the lower compartment in a ratio of 100 bacteria per cell. The chemotactic activities of the cell suspension alone and of the bacteria alone were also measured (Table 3). The presence of a cell suspension in the lower compartment did not produce a result significantly different ($P > 0.1$) from the saline control. The dead bacteria alone produced significantly less cell migration than was found with saline ($P < 0.05$). The cells and bacteria together produced a significant ($P < 0.02$) chemotactic response, (mean 3.4 times control (cells only) values, range 1.9-4.5).

In the presence of indomethacin (10 μg/ml - 28 μM) in three experiments cells and bacteria together had only slightly more chemotactic effect than saline controls. At a higher

Table 1 Chemotactic effect of prostaglandin E_1 (PGE$_1$) on polymorphonuclear (PMN) leucocytes

Expt no.	Lower chamber contents		Ratio of PGE$_1$ /saline
	Saline	PGE$_1$	
1	75	434	5.8
2	51	112	2.2
3	30	95	3.2
4	35	164	4.7
5	36	111	3.1
6	35	97	2.8
7	20	102	5.1
8	31	121	3.9
9	24	58	2.4
10	46	131	2.8
	$P < 0.02$ (paired t test)		mean = 3.6

Results of 10 experiments using Boyden chambers with different PMN leucocyte suspensions (3 x 10^6/ml) in the upper compartment and prostaglandin E_1 (1 μg/ml) or saline in the lower compartment. Figures give total cell counts in 9 high power fields on the lower surface of the filter.

concentration of indomethacin (280 µM) there was slightly less cell migration than in controls (2 experiments). Indomethacin did not affect the cell migration in the bacteria-free saline controls.

Phagocytosis of the bacteria by the PMN leucocytes was demonstrated by the uptake of ^{125}I-labelled human serum albumin (HSA) (Chang, 1969) from the incubation medium. PMN leucocytes incubated without bacteria showed no increase in uptake of [125I]-HSA over a 50 min period, while the [125I]-HSA content of the cells incubated with bacteria (100/cell) rose over the same period to over three times the initial value.

Prostaglandin production by polymorphonuclear leucocytes during phagocytosis

Several substrates were tried for phagocytosis: glass beads were too large for the PMN leucocytes to ingest. Microscopy showed that the cells tended to spread over the surface of the beads. Latex beads aggregated in solution at $37°C$, and the aggregated particles were too large for successful phagocytosis. In all experiments described here *Bordetella pertussis* was used. Microscopy confirmed that these were ingested by the PMN leucocytes.

Table 2 Chemotactic effect of different prostaglandins (PG) on polymorphonuclear (PMN) leucocytes

	10 ng/ml	100 ng/ml	1 µg/ml	10 µg/ml
PGE_1	1.62 ± 0.25	2.75 ± 0.42	3.09 ± 0.28	2.81 ± 0.63
n	7	7	22	10
P	<0.05	<0.01	<0.001	<0.02
PGE_2		0.45 ± 0.14	1.1 ± 0.55	1.2
n		7	5	2
P		<0.02	NS	
$PGF_{2\alpha}$		0.63 ± 0.2	1.24 ± 0.16	1.2
n		5	7	3
P		NS	NS	

Results using Boyden chambers with PMN leucocytes (3×10^6/ml) in the upper compartment and various concentrations of different prostaglandins in the lower compartment. Results are expressed as the ratio, experiment/control ± s.e.mean.
P values are those from Student's paired t test: NS indicates $P > 0.1$.

Table 3 Chemotactic effect of polymorphonuclear (PMN) leucocyte—bacteria mixtures

Expt. no.	Contents of lower compartment			
	Saline	Cell suspension	Dead bacteria	Cell suspension + dead bacteria
1	19	22	18	35
2	54	37	58	155
3	17	22	8	76
4	12	5	3	24
5	19	21	7	51
6	12	26	5	38
7	75	283	16	317

NS

$P < 0.05$

$P < 0.02$

Results (total cells in 9 high power fields) of 7 experiments using Boyden chambers with PMN leucocyte suspensions (3×10^6/ml) in the upper compartment, contents of the lower compartment as stated.
P values are those from Walsh test as shown for comparison with appropriate controls. NS : $P > 0.1$.
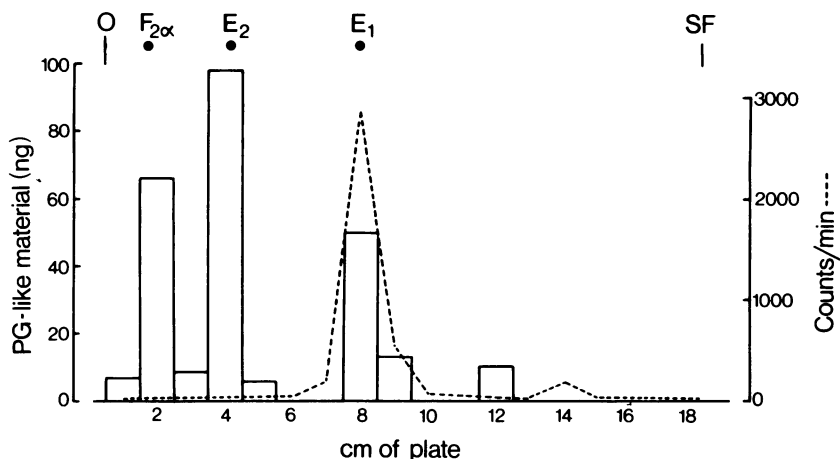
Figure 1 All thin-layer chromatography of prostaglandin (PG)-like material extracted from the supernatant of cell suspensions incubated with killed bacteria. The spots show the positions of standards of authentic prostaglandins $F_{2\alpha}$, E_2 and E_1. The broken line shows the radioactivity in successive samples eluted from the plate, to which ^{3}H-labelled prostaglandin E_1 had been applied with the sample. The histogram shows biological activity expressed as prostaglandin E_2 equivalents assayed on the rat stomach strip.

Initially the bacteria were used at a concentration of 1000 bacteria per cell. In these conditions there was negligible (less than 0.3 ng/10^7 cells) production of a prostaglandin-like material at zero-time, but production rose to 7.1 ng/10^7 cells (range, 4.3-11.0) after 1 h of incubation. In 5 experiments, the amount of prostaglandin-like material after 2 h ranged from 2.4 to 17.3 ng/10^7 cells (mean, 8.4). In general, amounts of prostaglandin-like material rose with time to a peak of over 30 ng/10^7 cells at 3 h, after which the level fell, presumably because destruction had overtaken release.

The amount of prostaglandin-like material in the supernatant also varied with the bacteria-cell ratio. When this ratio was between 0.1 and 10 there was a negligible amount of prostaglandin-like material found at 1 or 2 h (1 ng/10^7 cells). Above this ratio the level rose with the bacteria-cell ratio reaching a maximum at 1000, (range 4.3-17 ng/10^7 cells). There was no further increase at higher ratios.

In the presence of indomethacin (28 μM) the level of prostaglandin-like material in cell-bacteria mixtures was not significantly greater than in bacteria-free cell suspensions (5 experiments).

Extracts of supernatants of cell suspensions incubated with bacteria were subjected to thin-layer chromatography, using the AI system and measuring the activity eluted from the zones of the plate against prostaglandin E_2 standards on superfused rat stomach fundus strip. Sixty per cent of the recoverable activity chromatographed

with prostaglandin E_2, 25% with $F_{2\alpha}$, and the remainder was located in a position between prostaglandin $F_{2\alpha}$ and the origin. Further characterization of extracted prostaglandins using the AII system showed that 39-80% of the prostaglandin E-like activity corresponded to authentic prostaglandin E_1, both in its R_F value and in its biological activity on the three assay tissues. The rest of the activity had the chromatographic characteristics of prostaglandins E_2 and $F_{2\alpha}$.

Extracts from several experiments were combined to attain enough activity for accurate bioassay. [^{3}H]-prostaglandin E_1 was added before extraction to check that the mobility of the prostaglandin was not altered by the extraction procedure. Results of one of the chromatograms developed in the AII system are shown in Figure 1.

Prostaglandin synthesis by cell sonicates

In these experiments, the synthetic activity of the sonicates is expressed as ng of prostaglandin E_2-like material produced per mg protein in the sonicates. There was always some prostaglandin-like material present in the samples at zero-time; this is probably due to production during sonication. The activity found at zero-time was subtracted from that in subsequent samples to give a value representing net synthesis.

Without addition of substrate (2 experiments), there was a net synthesis after 30 min of 0.1 ng/mg protein. This synthesis rose in the presence of di-homo-γ-linolenic acid (10 μg/ml) to

Table 4   Effect of indomethacin on the synthesis of prostaglandin-like material by cell sonicates

Final conc. (μM) of indomethacin in incubation mixture	Prostaglandin-like material (ng/mg protein)	n
Control (no indomethacin)	3.0 ± 1.2	5
2.8	1.2	2
28	0.5 ± 0.4	4
280	0.2	3

Results are shown as the mean with s.e.mean.

0.5 ng/mg (2 experiments) and in the presence of arachidonic acid (10 μg/ml) (5 experiments) to 3.6 ± 1.2 ng/mg protein (mean ± s.e.mean).

In 8 experiments, cells were incubated with or without bacteria for 30 min before sonication. The cell sonicate from cells incubated without bacteria produced, after addition of arachidonic acid, 5.6 ± 1.8 ng prostaglandin E_2-like material/mg protein. Cells which had been preincubated with bacteria (100/cell) before sonication produced 10.6 ± 2.7 ng/mg protein. A Student's paired t test showed this increase to be significant ($P < 0.05$). Bacteria alone incubated for 30 min before sonication did not produce any prostaglandin-like material, and addition of bacteria to a cell sonicate immediately before incubation with substrate did not increase the prostaglandin production.

Indomethacin (0.2 ml of 1, 10 or 100 μg/ml in 20% absolute ethanol, 80% buffer) was added to the incubation mixture to give a final concentration of 2.8, 28 or 280 μM and the synthesis of prostaglandin-like material by cell sonicates, obtained from cells which had not previously been incubated, was measured. Increasing the concentration of indomethacin caused a progressive decrease in prostaglandin synthesis (Table 4). An equal amount of absolute ethanol added to the sonicates before incubation had no effect on the production of prostaglandin-like material. Addition of indomethacin to give a final concentration of 28 μM before the sonication of the cell suspension also caused a decrease in prostaglandin synthesis, to 0.1 ng/mg (mean of 3 experiments).

Discussion

Homogenates of rabbit PMN leucocytes have the capacity to synthesize prostaglandin from endogenous or exogenous precursors. The synthetase system of these cells is, like that of other tissues, inhibited by indomethacin. The finding that homogenates from cells which have been preincubated with bacteria have greater synthetic ability than homogenates from bacteria-free controls, suggests that the prostaglandin synthesis occurring during phagocytosis is not due only to increased substrate availability, or to cell membrane damage, but may involve a change in cellular enzyme activity during phagocytosis.

Rabbit peritoneal PMN leucocytes produce and release prostaglandins, including prostaglandin E_1, when ingesting dead bacteria. Furthermore, the amounts produced in vitro seem adequate to account for the levels of prostaglandins found in inflammatory exudates. Ten million cells produced up to 30 ng of prostaglandin-like activity in 3 hours. The concentrations attained are also adequate to account for the chemotactic influence of phagocytosing cells in vitro discussed below. Our findings strongly support the view of Movat, MacMorine & Takeuchi (1971) that the vascular permeability-increasing agent released from rabbit PMN leucocytes during phagocytosis is a prostaglandin. Experimental immunogenic uveitis has been studied in rabbits by Eakins et al. (1972). Prostaglandin E_1 was found in inflamed but not in normal rabbit eyes. Normal eye tissues contained only prostaglandins E_2 and $F_{2\alpha}$. The appearance of prostaglandin E_1 coincided with the invasion of the ocular tissues and aqueous humour by PMN leucocytes. These findings are consistent with our detection of E_1 among the prostaglandins produced by phagocytosing PMN leucocytes, and support our view that in some forms of inflammation the source of the prostaglandins found may be the PMN leucocytes.

We have confirmed the chemotactic activity of prostaglandin E_1 for washed (plasma-free) PMN rabbit leucocytes, first described by Kaley & Weiner (1971). We have further shown that this effect is concentration-dependent and detectable down to 10 ng prostaglandin E_1/ml, well below the total prostaglandin concentrations which have been reported in inflammatory exudates (80 ng/ml, Willis, 1969; 140 ng/ml, Eakins et al., 1972). It is thus possible that the prostaglandins in inflammatory lesions could have a chemotactic

effect *in vivo* on PMN leucocytes. The effect of prostaglandins on other motile cells such as macrophages was not studied, but seems worth investigating.

Phagocytosing PMN leucocytes, but not cells or bacteria alone, have a chemotactic effect on other cells. This chemotactic activity of cell-bacteria mixtures is greatly reduced or abolished in the presence of indomethacin (28 μM or 280 μM respectively). This supports the view that the effect may be mediated by a prostaglandin since at these concentrations indomethacin inhibits prostaglandin synthesis in such mixtures. Reports that indomethacin decreased cell motility (Phelps, 1969) are based on an experimental system in which urate crystals were used as a chemotactic stimulus. In the light of our findings the results can be re-interpreted as abolition by indomethacin of a prostaglandin-mediated chemotactic effect associated with phagocytosis of the crystals by the first cells reaching them.

It should be noted that the present studies were performed with washed cells, and that, *in vivo*, other chemotactic agents such as activated complement factors, may be more important than the prostaglandins. In addition, phagocytosing cells may release other substances which could act as chemotactic agents. This may explain why non-steroid anti-inflammatory drugs are unable to abolish fully the cellular invasion in inflammatory lesions.

Influences other than phagocytosis may also induce prostaglandin release from PMN leucocytes. Thrombin, for instance, does so (our unpublished observation) and this or other mechanisms may be concerned in chemotaxis in the absence of phagocytosis.

Moncada, Ferreira & Vane (1974) have shown that 3 h after carrageenin injection into the dog knee joint, prostaglandin E_2 can be detected in the synovial fluid. In human arthritis, however, where the PMN leucocyte concentration in synovial fluid is high, the predominant prostaglandin is E_1 (Higgs, Vane, Hart & Wojtulewski, 1974). Since PMN leucocyte migration begins in carrageenin-induced oedema 2-3 h after injection (Di Rosa, Papadimitriou & Willoughby, 1971), it seems possible that the source of the prostaglandin E_1 is the PMN leucocytes. In a similar model (Phelps & McCarty, 1967) indomethacin treatment resulted in decreased fluid exudate and reduced PMN leucocyte infiltration.

It is concluded that in some forms of inflammation, PMN leucocytes may be the source of the prostaglandin found. Such prostaglandin production, particularly that of prostaglandin E_1 could have a specific role in inducing or enhancing local migration of phagocytic cells, as well as in the modulation of the inflammatory effects of other mediators. Such a system would have the obvious advantage of encouraging invasion by phagocytic cells only as long as there was material capable of being phagocytosed present. It could not, alone, explain how such cellular migration is initiated.

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