

# Release of Prostaglandin by Mitogen- and Antigen-Stimulated Leukocytes in Culture

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**ABSTRACT** The prostaglandin (PG) content of mitogen- and antigen-stimulated leukocyte cultures was examined by a radioimmunoassay procedure employing an antiserum reactive with  $\text{PGB}_1$  and  $\text{PGB}_2$ , the alkaline dehydration products of  $\text{PGE}$  and  $\text{PGA}$ . At 48 h, mitogen-activated mouse spleen cell cultures showed 2–10-fold increases in the  $\text{PGE}$ , but not in the  $\text{PGA}$ , component of immunoreactive PG (iPG) fractionated by silicic acid column chromatography. Increases in iPG were detectable by h 16 in spleen cell cultures incubated with staphylococcal enterotoxin B. Since iPG levels rose only in the culture supernates and not in cells exposed to mitogens for 48 h, increases reflected extracellular release of PG. The validity of the radioimmunoassay determinations of  $\text{PGE}$  in spleen cell cultures was supported by the results of concomitant assessment of the  $\text{PGE}_2$  content of basal and enterotoxin-stimulated cultures by gas chromatography/mass spectrometry. By the latter method, the  $\text{PGE}_2$  content was three-fold higher in enterotoxin-activated, compared to basal, cultures at 48 h. Aspirin effectively suppressed increases in both iPG and  $\text{PGE}_2$ . In spleen cell cultures prepared from mice previously inoculated with an attenuated strain of yellow fever virus *in vivo* and then incubated with this virus *in vitro*, iPG levels increased two-fold over basal at 48 h. By contrast, iPG content of spleen cell cultures prepared from saline-inoculated mice was not appreciably altered by exposure to the virus *in vitro*.

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The enhancement of iPG release from cultured spleen cells by mitogens did not correlate with an ability of these agents to increase cellular cyclic AMP (cAMP) levels. Moreover, epinephrine and cholera toxin markedly increased spleen cell cAMP content but had no demonstrable effect on basal iPG levels, suggesting iPG release from these cells was not mediated by cAMP.

Incubation with mitogens also enhanced the iPG content of 72-cultures of human peripheral leukocytes and of human lymphocytes isolated by nylon chromatography. However, the iPG of cultures of human lymphocytes purified by glass bead chromatography and of mouse thymocytes was not appreciably altered when these cells were cultured with mitogens, even though DNA synthesis in both instances was markedly increased. Accordingly, iPG release was not an invariable concomitant of increased DNA synthesis in lymphoid cell cultures.

In summary, the results demonstrate that mitogen and antigen stimulation of leukocytes in culture may be accompanied by enhanced release of  $\text{PGE}$ . The mechanisms mediating this phenomenon and its biologic significance remain to be delineated, but participation of  $\text{PGE}$  in immunologically induced inflammatory responses seems possible.

## INTRODUCTION

Stimulation of cultured leukocytes with mitogens or antigens results in the release of soluble mediators with a wide variety of biologic actions (1, 2). Many of these agents appear to be proteins, while the chemical nature of others remains uncertain (1, 2). With regards to the latter, it is of interest that several of the biologic responses mediated by the supernates from stimulated

leukocyte cultures have also been shown to be induced by prostaglandins (PG's).<sup>1</sup> These common biologic effects include enhancement of vascular permeability, vasodilation, leukotaxis, and initiation of bone resorption (3-10). In addition, PG's have been implicated both as mediators and modulators of several inflammatory processes (4, 7, 11) and have been identified in a wide variety of inflammatory exudates (4, 12, 13). They may directly influence the function of both neutrophils and lymphocytes by activating the adenylate cyclase-cyclic adenosine 3',5'-monophosphate (cAMP) system of leukocytes (14-17). There is also evidence to suggest that release of PG's may occur in vivo in response to certain immunologic stimuli (4, 18, 19). However, the possibility that PG's are released by immunologically activated leukocytes in culture has not been specifically examined.

In view of the above observations and the fact that PG's are, in general, synthesized and released locally at their cellular sites of action (18, 20, 21), we assessed the PG content of mouse and human leukocyte cultures after mitogenic or antigenic stimulation in vitro.

## METHODS

**Preparation of cells.** Spleen and thymus cells from 5 to 10-wk-old inbred male C57BL/6 mice<sup>2</sup> and human peripheral leukocytes from healthy young adult male donors were studied. Suspensions of spleen or thymus cells were prepared by methods previously described (22, 23). Each organ was gently minced with scissors and the tissue fragments passed through a 60-mesh stainless steel screen into RPMI-1640 medium with 25 mM Hepes buffer.<sup>3</sup> These fragments were drawn through a 25-gauge needle to produce a suspension of single cells. The latter were then washed with buffered RPMI-1640, centrifuged at 200 *g*, and resuspended in the complete lymphocyte culture medium described below. Antigen-sensitive spleen cells were obtained by inoculating mice intraperitoneally with  $5 \times 10^5$  plaque-forming units of the 17D vaccine strain of yellow fever virus.<sup>4</sup> Mice were inoculated on day 0 and 7 with either virus or saline (controls), and spleens were harvested and studied on day 14 postinoculation.

Human peripheral leukocytes (approximately 60% granulocytes and 40% mononuclear cells) were isolated from freshly drawn heparinized blood by dextran sedimentation (24). Hypotonic lysis was employed to remove contaminating red cells. Purified human lymphocytes were prepared either by nylon chromatography or dextran-sedimented mixed leukocytes (24) or by glass bead chromatography (25). The former method yielded cell populations which were  $85 \pm 5\%$  mononuclear cells, while by the latter method, cell preparations consisted of 98-99% small lymphocytes

with 1-2% granulocytes and large mononuclear cells. Cultures of mouse and human leukocytes were always initiated on the day of cell preparation. As assessed by trypan blue exclusion, cell viability exceeded 95% at the initiation of the cultures and was  $81 \pm 5\%$  at the end of the culture periods.

**Method of lymphoid cell culture.** The techniques employed for lymphoid cell culture were modifications of those of Adler and Rabinowitz and Adler, Takiguchi, Marsh, and Smith (22, 23). Briefly, the complete culture medium consisted of RPMI-1640 with 25 mM Hepes buffer, 5% human serum (heat inactivated at 56°C for 30 min), 100 U/ml of penicillin,<sup>5</sup> and 100 mg/ml of streptomycin.<sup>6</sup> In culture of human cells, homologous serum was employed. All cultures were conducted in sealed tubes at 37°C with or without test agents, as indicated in Results. Mouse lymphoid cells were cultured for 48 h at a density of  $1.5 \times 10^7$  cells/3 ml of medium, while human cells were cultured for 72 h at a density of  $10^7$  mononuclear cells/3 ml.

**Incorporation of tritiated thymidine ( $[^3\text{H}]\text{Tdr}$ ).** Concomitant with the determination of prostaglandin content of the cultures in response to test agents, the effects of these agents on  $[^3\text{H}]\text{Tdr}$  incorporation into trichloroacetic acid precipitates of washed cells were also monitored in separate culture tubes. Unless otherwise indicated in Results,  $[^3\text{H}]\text{Tdr}$  (1  $\mu\text{Ci}/3$  ml)<sup>5</sup> was present for the last 24 of the 48-h (mouse lymphoid cells) or 72-h (human lymphocytes) culture periods, and its incorporation was assessed by previously described methods (23).

**Extraction and conversion of PG.** PG derived from the entire leukocyte culture, the culture supernate, or the resuspended leukocyte pellet was extracted by a two-step column chromatographic method. Cultures were acidified to pH 3 with 1 N HCl, homogenized, and poured onto 150  $\times$  8-mm columns containing 2.0 g of Amberlite XAD-2 resin (Mallinckrodt Chemical Works, St. Louis, Mo.). The columns were washed with 16 ml of distilled water, and PG was then eluted with 8.0 ml of methanol. Recovery of PG's from this step was at least 90% as monitored from the recovery of added tritium-labeled  $[^3\text{H}]\text{PGA}_1$ ,<sup>5</sup>  $[^3\text{H}]\text{PGE}_1$ ,<sup>5</sup> and  $[^3\text{H}]\text{PGF}_{1\alpha}$ .<sup>6</sup> This methanol eluate was evaporated and the residue then taken up in 1.0 ml of benzene:ethyl acetate:methanol (60:40:2 by volume). The latter was subjected to chromatography on a 150  $\times$  8-mm column containing 0.5 g of silicic acid (Bio-Rad Laboratories, Richmond, Calif.), added to the columns as a slurry (2 ml of 0.25 g/ml of benzene:ethyl acetate). PG's of A, E, and F types were then separated by the method of Jaffe, Behrman, and Parker (26) as follows: elution of PGA and B with 6 ml of benzene:ethyl acetate (60:40); elution of PGE with 12 ml of benzene:ethyl acetate:methanol (60:40:2); and elution of PGF with 3 ml of benzene:ethyl acetate:methanol (60:40:20). The efficiency of separation of the three fractions, assessed by adding  $[^3\text{H}]\text{PGA}_1$ ,  $\text{E}_1$ , or  $\text{F}_{1\alpha}$  just before chromatography, exceeded 90%. This value is consistent with previous findings (26).

After evaporation and resuspension of the three PG fractions in 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.4), each fraction was subjected to alkaline dehydration by adding 10  $\mu\text{l}$  of 1 N NaOH to the Tris suspension (final pH 11.5) and heating for 10 min at 95°C. This procedure converts PG's of the E and A type to type B (27). Overall recovery of

<sup>1</sup> Abbreviations used in this paper: cAMP, cyclic adenosine 3',5'-monophosphate; CON-A, concanavalin-A;  $[^3\text{H}]\text{Tdr}$ , tritiated thymidine; iPG, immunoreactive prostaglandin; PG, prostaglandin; PHA, phytohemagglutinin-P; SEB, staphylococcal enterotoxin B.

<sup>2</sup> The Jackson Laboratory, Bar Harbor, Maine.

<sup>3</sup> Grand Island Biological Co., Grand Island, N. Y.

<sup>4</sup> National Drug Co., Philadelphia, Pa.

<sup>5</sup> Tritiated thymidine (sp act 1.9 Ci/mmol) obtained from Schwarz/Mann Div., Becton, Dickinson and Co., Orangeburg, N. Y.

<sup>6</sup> Obtained from New England Nuclear, Boston, Mass.

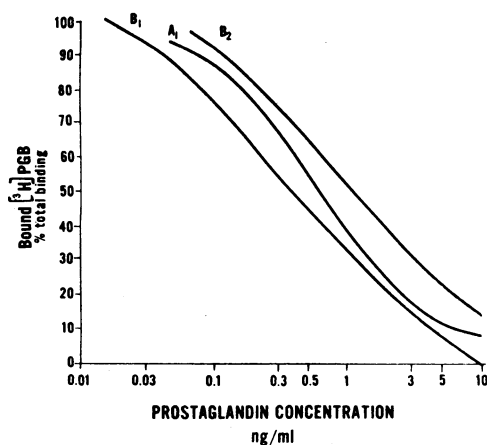


FIGURE 1 Cross-reactivity of anti-PGB<sub>1</sub> antiserum with PGA<sub>1</sub> and B<sub>2</sub>. Less significant cross-reactivity was observed with PGA<sub>2</sub>, E<sub>1</sub>, and E<sub>2</sub> (see text). A regression curve indistinguishable from that shown for PGB<sub>1</sub> was obtained when alkaline dehydrated PGE<sub>1</sub> was employed as the unlabeled antigen rather than commercial PGB<sub>1</sub>.

the immunoreactive PG in the E silicic acid fraction after extraction and conversion was monitored in each assay from the recovery of known amounts of unlabeled PGE<sub>1</sub><sup>7</sup> added to some control and stimulated culture tubes just before extraction. Mean recovery of added PGE<sub>1</sub> in 21 separate radioimmunoassays was  $79.1 \pm \text{SE } 5.2\%$ , with the PG values in every assay corrected for the recovery rate calculated for that assay.

**Radioimmunoassay of PG.** A commercially available antiserum to PGB<sub>1</sub><sup>8</sup> (anti-PGB<sub>1</sub>) was employed to estimate the PG content of the three PG fractions after alkaline dehydration. Initially, standard curves were prepared over a concentration range of 0.01–10 ng/ml, using identical concentrations of both commercially available PGB<sub>1</sub> and PGE<sub>1</sub> which had been subjected to alkaline dehydration as described above. Since these standards were found to yield analogous curves, only known concentrations of alkaline, dehydrated PGE<sub>1</sub> were then routinely employed; the unknowns and the standards to be assayed were subject to alkaline dehydration simultaneously.

The immunoassay reaction mixture consisted of 100  $\mu$ l of the standards (triplicates of each) or the unknowns (duplicates), 10  $\mu$ l of anti-PGB<sub>1</sub> antiserum, and 0.01  $\mu$ Ci of [<sup>3</sup>H]PGB<sub>1</sub> in 900  $\mu$ l of Tris-HCl buffer (pH 7.4). This mixture was incubated for 2 h at 37°C. At the end of this incubation, 1.0 ml of a 2.5% suspension of dextran-coated charcoal in Tris buffer (4°C) was added to each tube. Tubes were then incubated for 15 min at 4°C and spun at 2,000 rpm for 20 min to separate protein-bound (supernatant) from free [<sup>3</sup>H]PGB<sub>1</sub> (charcoal adsorbed). A 1.0-ml aliquot of the supernate was solubilized in a toluene-based scintillation solution (Scintisol/Scintilute, Isolab Inc., Akron, Ohio) and counted in a Nuclear Chicago Mark III liquid scintillation system (Nuclear Chicago Corp., Des Plaines, Ill.). Approximately 50% of the total added [<sup>3</sup>H]-PGB<sub>1</sub> was bound in the absence of unlabeled PGB<sub>1</sub>. Bound [<sup>3</sup>H]PGB<sub>1</sub> in samples containing known quantities of un-

labeled PGB<sub>1</sub> was expressed as a percentage of the total [<sup>3</sup>H]PGB<sub>1</sub> bound. Assay tubes without antiserum to PGB<sub>1</sub> were employed to correct for the nonspecific appearance of [<sup>3</sup>H]PGB<sub>1</sub> in the supernate after charcoal adsorption (2–3% of total added counts). With this assay system, 0.030 ng PGB<sub>1</sub> (or alkaline, dehydrated PGE<sub>1</sub>) could be reliably detected, and the regression curve behaved as a single exponential function between 0.030 and 3 ng of PGB<sub>1</sub> (Fig. 1).

**Gas chromatographic/mass spectrometric determination of PGE<sub>2</sub>.** PGE<sub>2</sub> content of the supernates from control and mitogen-stimulated mouse spleen cell cultures were determined by the general gas chromatographic/mass spectrometric method of Axen, Green, Horlin, and Samuelsson and Hammarström and Samuelsson (28, 29), except that preparative thin-layer chromatography, rather than reserved phase partition chromatography, was employed to purify PG extracts. The supernates from 10 spleen cell culture tubes ( $1.5 \times 10^7$  cell/3 ml medium) were pooled for a single determination of PGE<sub>2</sub> content by gas chromatography/mass spectrometry. These measurements were most generously performed by Dr. Leon Wolfe of the Montreal Neurologic Institute, Montreal, Canada.

**Determination of mouse spleen cell cAMP content.** To assess the effects of test agents on cellular cAMP content, leukocyte cultures were centrifuged at 1,500 rpm for 2 min at 4°C and the cell pellets immediately homogenized in 300  $\mu$ l of hot sodium acetate buffer (pH 4.0, 50 mM) which contained 2,000 cpm of [<sup>3</sup>H]cAMP<sup>9</sup> to assess recovery. These homogenates were heated for an additional 10 min at 95°C and centrifuged at 3,500 rpm for 10 min. Aliquots (50 and 25  $\mu$ l) of the supernates were then assayed directly for cAMP content by using the protein-binding method of Gilman (30). Validity of the cAMP assay was confirmed by the linearity of sample dilutions and destruction of measureable cAMP with cyclic phosphodiesterase.

**Test substances.** Mouse and human lymphoid cells were treated with one or more of the following test agents at doses indicated in Results: (a) staphylococcal enterotoxin B (SEB) purified by the general method of Schantz et al. (31) and kindly supplied by Dr. J. D. Metzger, U. S. Army Medical Research Institute for Infectious Diseases, Frederick, Md.; (b) phytohemagglutinin P (PHA), Difco Laboratories, Detroit, Mich.; (c) concanavalin A (CON-A), Pharmacia Fine Chemicals, Piscataway, N. J.; (d) aspirin, U.S.P., Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.; (e) 17D strain of yellow fever virus<sup>8</sup>; (f) cholera toxin, kindly supplied by Dr. R. Rappaport, Wyeth Laboratories, Marietta, Pa.; (g) bovine serum albumin, fraction V, Sigma Chemical Co., St. Louis, Mo.; and (h) l-epinephrine bitartrate, Calbiochem, San Diego, Calif.

Statistical significance of differences between mean values was analyzed by using Student's *t* test for unpaired values.

## RESULTS

**Cross-reactivity of anti-PGB<sub>1</sub> antiserum.** As shown in Fig. 1, [<sup>3</sup>H]PGB<sub>1</sub> was most effectively displaced from the anti-PGB<sub>1</sub> antiserum by PGB<sub>1</sub> (or alkaline, dehydrated PGE<sub>1</sub>). However, significant displacement was also observed with PGA<sub>1</sub> and PGB<sub>2</sub> (Fig. 1). Approximately 5 ng/ml of PGA<sub>2</sub>, 10 ng/ml of PGE<sub>1</sub>, and 80 ng/ml of PGE<sub>2</sub> were required to displace 50% of [<sup>3</sup>H]PGB<sub>1</sub> from the antiserum, quantities at least 10-

<sup>7</sup> Generous gift of Dr. John Pike, Upjohn Co., Kalamazoo, Mich.

<sup>8</sup> Clinical Assays, Cambridge, Mass.

TABLE I  
*iPG Content of the PGE and PGA Silicic Acid Fractions from Mitogen-Stimulated  
Mouse Lymphoid Cell Cultures*

Test agent	Dose	Culture extract	iPG content of silicic acid fraction		[ <sup>3</sup> H]Tdr incorporation
			PGE	PGA	
	$\mu\text{g/ml}$		$\text{ng}$		$\text{cpm}/1.5 \times 10^6 \text{ cells}$
None	—	C + M	$0.71 \pm 0.03$	$0.65 \pm 0.07$	$1,496 \pm 121$
Aspirin	30	C + M	$0.42 \pm 0.04^*$	$0.38 \pm 0.04^*$	$1,055 \pm 140$
SEB	75	C + M	$3.97 \pm 0.20^*$	$0.90 \pm 0.12$	$43,214 \pm 2,324^*$
SEB + (aspirin)	75 + (30)	C + M	$1.72 \pm 0.12^{*\dagger}$	—	$33,542 \pm 1,056^*$
PHA	5§	C + M	$3.53 \pm 0.16^*$	$0.81 \pm 0.15$	$50,897 \pm 1,542^*$
CON-A	10	C + M	$2.57 \pm 0.20^*$	$0.87 \pm 0.11$	$61,523 \pm 1,287^*$
BSA	75	C + M	$0.72 \pm 0.03$	—	$1,587 \pm 96$
None	—	C	$0.39 \pm 0.03$	—	—
SEB	75	C	$0.43 \pm 0.03$	—	—
None	—	M	$0.56 \pm 0.12$	—	—
SEB	75	M	$2.68 \pm 0.35^*$	—	—
Thymus cell cultures					
None	—	C + M	$0.42 \pm 0.07$	—	$95 \pm 12$
SEB	75	C + M	$0.41 \pm 0.09$	—	$2,658 \pm 152^*$
CON-A	10	C + M	$0.50 \pm 0.08$	—	$3,122 \pm 259^*$

Effects of aspirin, SEB, PHA, CON-A, and bovine serum albumin (BSA) on the iPG content of spleen and thymus cell cultures derived from the same mouse donors. After a 48-h incubation, the cell pellet (C) alone, culture medium (M) alone, or the entire culture (C + M) was extracted for iPG content. Values represent mean  $\pm$  SE, with an  $n$  of 5 for iPG and 3 for [<sup>3</sup>H]Tdr incorporation.

\* Difference from control significant at  $P < 0.01$ .

† Difference from SEB alone significant at  $P < 0.01$ .

§ The dose of PHA refers to  $\mu\text{l}$  of reconstituted commercial extract/ml of culture medium.

fold greater than needed to achieve similar displacement with  $\text{PGB}_1$  (not shown). No detectable displacement of [<sup>3</sup>H] $\text{PGB}_1$  was observed with 100 ng of the prostaglandin precursors, linoleic and arachidonic acids, 100 ng of  $\text{PGF}_{1\alpha}$  or  $\text{F}_{2\alpha}$ , or with extracted alkaline, dehydrated complete culture medium in the absence of cells. In addition, 100 ng of 15-keto- $\text{PGE}_1$  displaced less than 10% of [<sup>3</sup>H] $\text{PGB}_1$  (not shown). Since the antiserum employed was clearly not monospecific for  $\text{PGB}_1$  (Fig. 1), experimental values for the PG content of leukocyte cultures are reported only as the immunoreactive PG (iPG) found in each of the silicic acid fractions.

*PG content of mitogen-stimulated mouse lymphoid cell cultures.* As shown in Table I, the iPG content of the PGE silicic acid fraction from 48-h mouse spleen cell cultures stimulated with the mitogens SEB, PHA, and CON-A was significantly increased. In four separate experiments, the magnitude of this increase in mitogen-stimulated cultures ranged from 2- to 10-fold over control. No significant alterations in the iPG content of the PGA (Table I) or PGF (not shown) silicic acid fractions from stimulated cultures were detectable with the immunoassay method employed. As indicated in

Table I, mouse spleen cell [<sup>3</sup>H]Tdr incorporation was concomitantly monitored and was markedly potentiated by all of the mitogens employed. The addition of a nonmitogenic protein, bovine serum albumin, to cultures altered neither iPG content nor [<sup>3</sup>H]Tdr incorporation, whereas the addition of aspirin suppressed both basal iPG and the increase in iPG observed in response to SEB (Table I). In agreement with previous observations (32), the dose of salicylate employed in the present study (30  $\mu\text{g/ml}$ ) did not adversely affect cell viability as assessed by trypan blue exclusion at the end of the culture period, although [<sup>3</sup>H]Tdr incorporation was modestly reduced.

As also shown in Table I, the increase in iPG content of SEB-treated spleen cell cultures was attributable to release of PG into the culture medium, since the content of the latter, but not of the cell pellet, was enhanced after stimulation with SEB. Similar results were obtained with PHA and CON-A. Addition of mitogens alone in the absence of added spleen cells did not detectably influence the iPG content of the culture medium (not shown). Further, SEB and CON-A failed to increase the iPG of either the PGE or A silicic acid fraction from mouse thymus cell cultures. However,

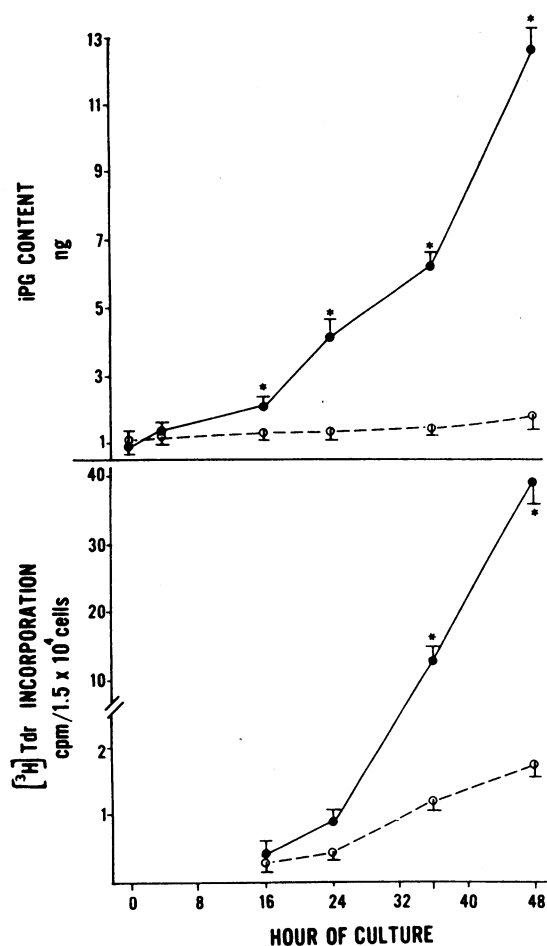


FIGURE 2 Time-course of alterations in the iPG content and [<sup>3</sup>H]Tdr incorporation of control mouse spleen cell cultures (open circles) and cultures treated with 75 µg/ml of staphylococcal enterotoxin B (solid circles). Values represent mean±SE with an *n* of 5 for iPG and 4 for [<sup>3</sup>H]Tdr incorporation. Combined cell pellet and medium were extracted for iPG. [<sup>3</sup>H]Tdr was added at h 0; \* indicates *P* < 0.01 compared to control value at the same hour.

[<sup>3</sup>H]Tdr incorporation by thymus cells exposed to these mitogens was clearly increased (Table I).

Fig. 2 depicts the time-course of changes in the iPG content of spleen cell cultures stimulated with SEB. A significant increase in the iPG content of the PGE silicic acid fraction was noted as early as 16 h after the initiation of the cultures. At this time, moderate increases in [<sup>3</sup>H]Tdr incorporation (<two-fold over control) were observed in cells exposed to SEB. Comparable results were obtained when [<sup>3</sup>H]Tdr incorporation was assessed sequentially in SEB-stimulated cells incubated with the labeled nucleotide for 3 h before harvest rather than for the entire culture period (increase in [<sup>3</sup>H]Tdr incorporation over control, two-fold at 16 and 24 h, 13-fold at 36 h, and 17-fold at 48 h).

PG content of antigen-stimulated mouse spleen cell cultures. As shown in Table II, spleen cell cultures prepared from mice with previous *in vivo* exposure to yellow fever virus demonstrated enhancement of both the iPG content of the PGE silicic acid fraction and of [<sup>3</sup>H]Tdr incorporation upon *in vitro* incubation with this virus. By contrast, concomitantly studied spleen cells from saline-inoculated mice did not respond *in vitro* to the virus, although both the iPG content and [<sup>3</sup>H]Tdr incorporation of these cultures were increased by SEB. The iPG content of the PGA silicic acid fraction of cultures was not detectably altered by *in vitro* exposure to yellow fever virus of spleen cells obtained from either sensitized or control mice (not shown).

*Determination of PGE<sub>2</sub> by gas chromatography/mass spectrometry.* Table III compares changes in the iPG content of the PGE silicic acid fraction of supernates from spleen cell cultures to alterations in the PGE<sub>2</sub> content of these supernates as determined by gas chromatography/mass spectrometry. A clear increase of comparable magnitude occurred in both these parameters in cultures stimulated with SEB. Increases in both parameters were suppressed by aspirin (Table III). In view of the differences in methodology and the demonstrated cross-reactivity of the antiserum employed in the immunoassay procedure (Fig. 1), the close correspondence in the absolute values obtained is likely coincidental. However, the results suggest that the increase in iPG in SEB-stimulated cultures may,

TABLE II  
iPG Content of Antigen-Stimulated Mouse Spleen Cell Cultures

Mouse donor	In vitro test agent	iPG content	[ <sup>3</sup> H]Tdr incorporation
		ng	cpm/1.5 × 10 <sup>6</sup> cells
Saline-inoculated	None	1.13±0.10	1,951±102
	YFV	1.02±0.03	2,110±86
	SEB	5.14±0.41*	21,562±1,015*
YFV-inoculated	None	1.16±0.04	1,168±89
	YFV	2.04±0.29*	8,993±251*
	SEB	3.14±0.39*	37,535±1,433*

Mice were inoculated with either saline or 5 × 10<sup>5</sup> plaque-forming units (PFU) of an attenuated strain of yellow fever virus (YFV) at 14 and again at 7 days before the *in vitro* study. Spleen cells obtained from these mice were then cultured with YFV (10<sup>4</sup> PFU/3 ml) or with 75 µg/ml of SEB. The entire cultures were extracted for determination of iPG content of the PGE silicic acid fraction. Values represent mean±SE with an *n* of 8 for iPG and 4 for [<sup>3</sup>H]Tdr incorporation.

\* Difference significant from the corresponding control at *P* < 0.01.

TABLE III  
Determination by Gas Chromatography/Mass Spectrometry  
(GC/MS) of PGE<sub>2</sub> Release by Cultured  
Mouse Spleen Cells

Test agent	GC/MS-PGE <sub>2</sub> ng released/1.5 × 10 <sup>7</sup> cells/3 ml medium	iPG
None	1.76, 1.59	1.05 ± 0.12
SEB	4.79, 5.20, 5.39	4.29 ± 0.12*
SEB + aspirin	1.69, 2.65	0.66 ± 0.03‡

The individual GC/MS determinations of PGE<sub>2</sub> content shown were each made on supernates pooled from 10 separate culture tubes containing 1.5 × 10<sup>7</sup> spleen cells in 3 ml of medium. iPG of the E silicic acid fraction represents the mean ± SE of determinations on 5 culture tubes from the same experiment. Doses of test agents employed were as indicated in Table I.

\*  $P < 0.01$  compared to control.

‡  $P < 0.01$  compared to SEB alone.

at least in part, be due to enhanced release of PGE<sub>2</sub>. Alterations in the PGE<sub>1</sub>, A<sub>1</sub> or A<sub>2</sub> content of the cultures could not be specifically determined by gas chromatography/mass spectrometry. However, preliminary findings with this method have suggested an increase in the PGF<sub>2</sub> content of mitogen-stimulated cultures (not shown).

**cAMP content of spleen cells.** Spleen cell cAMP content (mean basal ± SE, 9.2 ± 0.6 pmol/10<sup>7</sup> cells) was significantly enhanced by 5 µg/ml epinephrine (16.5 ± 1.3 at 10 min) and 5 µg/ml of cholera toxin (123.6 ± 9.4 at 1 h) but was not altered by SEB, CON-A, or PHA. By contrast, neither epinephrine nor cholera toxin detectably influenced basal iPG release by spleen cells when the latter was assessed at 10 min, 1 h, or 48 h of culture. Further, incubation of spleen cells with both SEB and cholera toxin suppressed iPG release at 48 h (mean ± SE, 0.91 ± 0.10 ng released/1.5 × 10<sup>7</sup> cells/3 ml culture medium) compared to that observed in response to SEB alone (3.25 ± 0.30),  $P < 0.01$ . The latter effect of cholera toxin on SEB-stimulated cultures was accompanied by a 72% reduction in [<sup>3</sup>H]Tdr incorporation.

**PG content of human peripheral leukocyte and lymphocyte cultures.** As shown in Table IV, the iPG content of the PGE silicic acid fraction of cultures of mixed human leukocyte populations (approximately 60% granulocytes and 40% mononuclear cells) was increased by mitogenic stimuli. This increase in iPG was suppressed by aspirin, which in the dose employed (30 µg/ml) also resulted in a slight decrease in [<sup>3</sup>H]Tdr incorporation. The iPG content of culture of human peripheral lymphocytes, prepared by nylon column chromatography, was also enhanced, but the mag-

nitude of the change was less than that observed with heterogeneous leukocyte preparations (Table IV). By contrast, mitogens did not detectably influence the iPG content of cultures of lymphocytes which had been purified by glass bead chromatography. However, [<sup>3</sup>H]-Tdr incorporation by these lymphocytes was clearly potentiated by mitogens (Table IV). The cells employed in the three experiments shown in Table IV were isolated from separate donors, but similar results were observed when each experiment was repeated twice using lymphocytes from different donors. The iPG content of the PGA fraction of stimulated human leukocyte or lymphocyte cultures was not significantly different from that of control cultures (not shown).

## DISCUSSION

The results indicate that the PGE content of leukocyte cultures is enhanced by mitogenic and antigenic stimulation. Using an antiserum reactive with both PGB<sub>1</sub> and B<sub>2</sub>, the alkaline dehydration products of PGE<sub>1</sub> and E<sub>2</sub>, increased quantities of immunoreactive prostaglandin were detected in the alkaline, dehydrated PGE silicic acid fractions from both stimulated mouse and human leukocyte cultures. Moreover, by gas chromatographic/mass spectrometric analysis a specific increase in the PGE<sub>2</sub> content of SEB-stimulated mouse spleen cultures

TABLE IV  
iPG Content and [<sup>3</sup>H]Tdr Incorporation of Human Peripheral  
Leukocyte and Lymphocyte Cultures

Test agent	Dose µg/ml	iPG ng	[ <sup>3</sup> H]Tdr incorporation cpm/10 <sup>6</sup> mononuclear cells
Expt I. Mixed leukocytes			
None	—	0.27 ± 0.03	158 ± 43
SEB	15	4.29 ± 0.36*	7,374 ± 572*
PHA	2‡	2.62 ± 0.15*	31,018 ± 3,538*
CON-A	10	2.09 ± 0.20*	33,628 ± 1,108*
SEB + (aspirin)	15 + (30)	2.08 ± 0.17§	6,372 ± 1,018*
Expt II. Nylon column purified lymphocytes			
None	—	0.20 ± 0.03	85 ± 9
SEB	15	0.57 ± 0.04*	9,553 ± 1,613*
CON-A	10	1.12 ± 0.10*	3,295 ± 378*
SEB + (aspirin)	15 + (30)	0.31 ± 0.05§	6,314 ± 648*
Expt III. Glass bead column purified lymphocytes			
None	—	0.30 ± 0.02	15 ± 1
SEB	15	0.37 ± 0.09	4,406 ± 507*
CON-A	10	0.31 ± 0.03	1,672 ± 146*

Culture tubes contained 10<sup>7</sup> mononuclear cells/3 ml medium and were incubated for 72 h. iPG content of the PGE silicic acid fraction are shown; the entire culture was extracted. Values represent mean ± SE with an  $n$  of 5 for iPG and 3 for [<sup>3</sup>H]Tdr incorporation. Abbreviations of test agents are as indicated in Table I.

\* Difference from corresponding control significant at  $P < 0.001$ .

‡ Dose of PHA refers to µl of reconstituted commercial extract/ml of culture medium.

§ Difference from SEB alone significant at  $P < 0.01$ .

was observed, which corresponded in magnitude to the increase in the iPG content of the PGE silicic acid fraction of these cultures. By contrast, no significant increase in the iPG content of the PGA silicic acid fractions of stimulated leukocyte cultures was noted following alkaline dehydration, even though the latter procedure is known to convert  $\text{PGA}_1$  and  $\text{A}_2$  to  $\text{PGB}_1$  and  $\text{B}_2$  (27). The antiserum employed in the present study demonstrated little cross-reactivity with  $\text{PGF}_{1\alpha}$  or  $\text{F}_{2\alpha}$ . Accordingly, the possibility of an alteration in the PGF levels of stimulated cultures is not excluded by the radioimmunoassay findings, and, indeed, preliminary assessment of  $\text{PGF}_{2\alpha}$  content by gas chromatography/mass spectrometry suggests that this moiety may also be increased in mitogen-stimulated mouse spleen cell cultures.

Separate analysis of the iPG content of the leukocyte pellets and of the culture supernates after mitogenic stimulation (Table I) demonstrated that the elevated iPG observed in extracts from whole cultures was owing entirely to an increase in media iPG, a finding which implies extracellular release of PG by stimulated leukocytes. The ability to suppress the iPG content of stimulated cultures with aspirin, an inhibitor of prostaglandin synthesis (33), not only supports the validity of the iPG determinations but also indicates that the increases in prostaglandin were mediated, at least in part, by *de novo* cellular synthesis. These findings are consistent with previous studies demonstrating that prostaglandins are not stored in cells but formed immediately before release, probably from fatty acid precursors derived from the phospholipids of cell membranes (21).

It is tempting to speculate that enhanced synthesis and release of PG's in stimulated leukocyte cultures may be a function of the activated lymphocytes in these cultures. The 16-h latent period between exposure of spleen cell cultures to mitogens and detection of a significant increase in iPG content (Fig. 2) suggests the need for a preliminary phase of cell activation. In addition, marked suppression of mitogen-induced mouse spleen cell activation by cholera toxin was associated with a clear decrease in the iPG content of these cultures. However, activation of lymphoid cells by mitogens, as reflected by enhanced [ $^3\text{H}$ ]Tdr incorporation, was not uniformly accompanied by release of increased quantities of iPG. Activation of mouse thymocytes and glass bead-purified human lymphocytes by mitogens did not significantly increase the iPG content of these cultures (Tables I and IV). Although a precise explanation is lacking at present, several factors might account for these findings: (a) activated thymocytes and purified human lymphocytes may release types of PG not detected by the radioimmunoassay procedure employed, such as  $\text{PGF}_{1\alpha}$  or  $\text{F}_{2\alpha}$ ; (b) cells other than lymphocytes, such as granulocytes or macrophages, may mediate release of iPG in cultures of spleen cells or mixed human leukocytes; and

(c) iPG release may depend upon an interaction between activated lymphocytes and other leukocytes in culture. The appropriate cooperative cells may be absent from populations of thymocytes and glass bead-purified human lymphocytes. There is precedence for in vitro cooperativity between stimulated lymphocytes and glass-adherent cells, such as macrophages, in the mediation of other biologic actions of lymphocytes (34–36). Whether this in fact accounts for the failure to observe iPG release from some populations of activated lymphoid cells remains conjectural.

In a number of tissues, increased intracellular cAMP levels are thought to initiate release of PG's including those of the E type (37–39). However, in cultured leukocytes release of PGE did not appear to be mediated by cAMP. Mitogenic agents effective in enhancing PGE release did not detectably influence the cAMP levels of mouse spleen cells. Moreover, cholera toxin and epinephrine, agents which markedly increased lymphoid cell cAMP content, did not stimulate prostaglandin release. Although cholera toxin is thought to initiate prostaglandin release from certain tissues, such as intestinal mucosa (37, 40), this agent actually suppressed mitogen-induced release of iPG from lymphocytes. The latter action of cholera toxin is quite likely related to cAMP-mediated inhibition of lymphoid cell activation (41, 42). Since prostaglandins appear to be derived from cell membrane phospholipid (21), it is possible that mitogens induce release of these agents through effects on membrane phospholipid metabolism (43), rather than through cAMP. In this regard, it has been suggested that activation of phospholipase may be an initial action of agents which trigger the release of PG (21).

The physiologic significance of the release of PGE by leukocytes upon stimulation in vitro is uncertain. Cultured human and mouse cells other than leukocytes have also been shown to release PG's (29, 44, 45), and release of these agents by some types of neoplastically transformed cells appears to be accentuated (44, 46, 47). Accordingly, enhanced release of prostaglandin does not qualify as a response which is unique to immunologically stimulated leukocytes in culture. Nevertheless, in view of the known influences of these agents on inflammatory and immune processes (4, 11), it seems possible that release of specific PG's from immunologically activated leukocytes might be of functional consequence. For example,  $\text{PGE}_1$  or  $\text{E}_2$  appear capable of mediating certain of the biologic actions known to be associated with the supernates from mitogen- or antigen-activated lymphoid cell cultures. In particular,  $\text{PGE}_1$  and  $\text{E}_2$  both enhance vascular permeability (4, 5) and the resorption of bone (10). Supernates from stimulated cultures also induce these same responses (1–3), and the soluble mediators responsible have not been well characterized. Alternatively, the PGE released by im-

munologic stimuli might ultimately act to modulate an ensuing inflammatory response. At high doses, PGE<sub>1</sub> and E<sub>2</sub> have been shown to inhibit the morphologic transformation and cytotoxic activity of lymphocytes (16, 17, 42), as well as lysosomal enzyme and histamine release from other leukocytes (15, 48). By such actions, locally released PGE, if present in sufficient quantity, might participate in a negative feedback inhibition system acting to limit the inflammatory process.

It is clear that delineation of the biologic significance of the release of prostaglandin from immunologically activated leukocytes in culture will require more precise quantitation of the specific prostaglandin moieties released and evidence that similar events occur in vivo. The results of the present study suggest that a further evaluation of this response is warranted.

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