Prostaglandin E_2 biphasic control of lymphocyte proliferation: inhibition by picomolar concentrations

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Abstract Prostaglandins (PGs) have an important physiological role in the modulation of various cell immune functions. The main sources of PGs during immune responses are monocyte cells. We report here the ability of non-stimulated macrophages to synthesize prostanoids and show that peritoneal mouse macrophages synthesize PGE_2, PGF_{2a} and thromboxane B_2, spleen macrophages produce PGE_2 and PGF_{2a}, and in a fresh medium this synthesis reaches a constant basal level in a few hours. We studied the kinetics of Con A-induced proliferation of murine splenocytes under the influence of a wide range of PGE_2 concentrations (10^{-14}–10^{-7} M). The suppressive effect of PGE_2 decreased when its concentration was lowered and disappeared at 10^{-9} M PGE_2 (this concentration corresponded to the basal level of non-stimulated macrophage synthesis of PGE_2). Further lowering of the concentration became essential for the proliferation process once again, and at picomolar concentrations PGE_2 caused a suppressive effect comparable with that for 10^{-8} M PGE_2. We also found that PGE_2 significantly inhibited cell proliferation when it was added 1 h before the addition of mitogen, as compared with simultaneous mitogen addition. The effect was obtained for both low (10^{-12} M) and high (10^{-8} M) PGE_2 concentrations. This phenomenon of PGE_2 biphasic control of lymphocyte proliferation may play an important role in cellular homeostasis, in particular in immune cell function regulation.

Key words: Prostaglandin E_2; Adaptation; Macrophage; Splenocyte; Proliferation

1. Introduction

Prostaglandins (PGs) of the E series are recognized to play an important regulatory role in inflammation and immune responses [1,2]. PGE_2 increases the expression of receptors on lymphocytes, promotes immunoglobulin synthesis, inhibits interleukin production, cytotoxicity and lymphocyte proliferation [1,3,4]. The broad range of PGE_2 biological actions is mediated through its binding to specific plasma membrane receptors [5]. Based on the affinity to receptor-specific agonists and antagonists and on their association with different intracellular signaling pathways, the PGE_2 receptors are divided into subclasses [4,6,7]. Thus PGE_2 can modulate cell responses through multiple pathways.

While lymphocytes themselves do not synthesize PGE_2, non-lymphoid inhabitants of the lymphocyte microenvironment such as macrophages, follicular dendritic cells, fibroblasts, and vascular endothelial cells produce PGE_2. This production dramatically increases in response to a variety of immunological and inflammatory stimuli (mitogens, cytokines, or tumor promoters) [3], and so much attention is paid to the analysis of PGE_2 effects under these conditions. Meanwhile, it is known that the key enzyme of PG synthesis, prostaglandin H synthase (PGHS) [8], exists in two isoforms, PGHS-1 and -2. PGHS-1 is usually described on non-stimulated cells and referred to as the 'constitutive' form of PGHS. Accordingly, PGHS-2 is referred to as the 'inducible' form of PGHS, as it is not expressed in most cells and tissues and it can be induced in cells treated with inflammatory stimuli [9]. The existence of a constitutive form of PGHS indicates that the level of PG synthesis by non-stimulated cells (basal level) may be important in a long-term regulation process. Previously we have shown that the basal level of arachidonic acid (AA) metabolites on non-stimulated peritoneal macrophages is modulated by opioid substances like morphine [10] or by an inhibitor of PGHS, ibuprofen [11]. There was also an observation that basal PG synthesis depended upon the time of macrophage cultivation [12]. Despite these data it is still unknown what role, if any, prostanoid synthesis by non-stimulated macrophages plays in the cellular immune response. In order to find out any correlation between basal prostanoid synthesis by macrophages and mitogen-stimulated lymphocyte proliferation we investigated the mouse splenocyte proliferation system and showed that both increase and decrease of the basal PGE_2 level leads to the suppression of lymphocyte proliferation, whereas at PGE_2 concentrations corresponding to the basal PGE_2 level the lymphocyte proliferation was not changed.

2. Materials and methods

2.1. Cell cultures

Macrophages and splenocytes were obtained from 5–6 week old CBA male mice. The mice were killed by cervical dislocation. The macrophages were collected from five or six untreated mice by peritoneal lavage with Dulbecco's modified Eagle's medium (DMEM, Sigma), supplemented with 2 mM L-glutamine (Sigma), sodium bicarbonate and penicillin-streptomycin (2% solution). After several washes the cells ($1.2–1.8\times10^6$/ml) were plated out on culture dishes and incubated for 2–3 h with 10% heat-inactivated fetal calf serum (FCS, Flow) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 to allow adherence. Then non-adherent cells were removed and fresh medium was added to the macrophage monolayers. For splenocyte preparation a spleen was put into PBS, minced, centrifuged at $300\times g$ for 10 min and resuspended in complete RPMI 1640 medium (Serva) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 mM HEPES, sodium bicarbonate and penicillin-streptomycin (2% solution). Cell viability was estimated by the trypan blue test and was never below 96%.

2.2. Turnover of [^{3}H]arachidonic acid in macrophages

Adherent macrophages in 96-well plates were cultured in fresh DMEM supplemented with 2% FCS and with the addition of 25 nCi of [^{3}H]AA (186 Ci/mmol). After 4 h the macrophages were

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washed three times with phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA, Serva). Then the cells were incubated in fresh DMEM with 0.5% BSA for different time intervals. The radioactivity of supernatants was determined by standard liquid scintillation counting.

2.3. High-pressure liquid chromatography of prostanoids

Adherent macrophages in 24-well plates were cultured in fresh DMEM supplemented with 0.5% BSA. Splenocytes in 6-well plates were cultured in complete RPMI 1640 (4×10^6/ml) and at the chosen time cell supernatants were collected and gently centrifuged. Prostanoids synthesized by the cells were determined as derivatives of 4-bromomethyl-7-methoxycoumarin (BrMMC, Sigma) by HPLC with fluorescent detection as described previously [13]. Briefly, cell supernatants were acidified with 1 M HCl to pH 3.0 prior to extraction with ethyl acetate. The extracts were left overnight with sodium sulfate. The solvent was evaporated to dryness under a stream of nitrogen. The residues were dissolved in acetonitrile and stored at −20°C. The BrMMC (4 mM, 50 μl in acetonitrile) and potassium carbonate (1 mg) were added to the samples, vortexed and kept at 50°C for 15 min in the darkness. The samples were applied to a reverse-phase HPLC column C_{16} ('Diasorb'-130 T, 6 μm, 250×4 mm) and eluted with acetonitrile-water (1:1, v/v), the flow rate was 0.8 ml/min. The excitation wavelength was set at 313 nm, the emission wavelength at 370 nm. For calibration authentic standards were used. Retention times were 11, 17 and 29 min for PGE_2, PGF_{2a} and TxB_2 respectively. The detection limit was in the range of 0.1–3.0 ng depending on the prostanoid type.

2.4. Mitogen-induced proliferation

Splenocytes in 96-well plates were cultured in complete RPMI 1640 (initial cell concentration 10^6/ml) for different time periods. The final concentration of concanavalin A (Con A, Fluka) was 2 μg/ml. The effects of the concentration of PGE_2 on cell proliferation were investigated in 12 independent experiments. For these experiments PGE_2 and Con A were added to the cells simultaneously. The effect of PGE_2 depending on the time of its addition was studied in three independent experiments. PGE_2 at 10^{-8} M and 10^{-12} M was added 1 h before initiating the proliferation and also simultaneously with mitogen addition. During the last 24 h the cells were pulsed with [^{3}H]thymidine (25 Ci/mmol, final concentration 5 μCi/ml). Then the cells were harvested on paper filters and the amount of [^{3}H]thymidine incorporated into the cells was measured by standard liquid scintillation counting. The results shown are the means of triplicate determinsations which varied by less than 7%.

3. Results and discussion

3.1. PG synthesis by non-stimulated peritoneal and spleen macrophages

We examined the ability of non-stimulated peritoneal macrophages to synthesize prostanoids during cell cultivation. The cells were prelabeled with [^{3}H]AA, and the kinetics of radioactive product release during the macrophage cultivation was studied. The extracellular release of labeled [^{3}H]AA and its metabolites began 1 h after the addition of fresh medium, reached a constant level in 4 h and then remained constant for 12 h at least (Fig. 1). Based on these results we selected the time points for HPLC detection of PGs. It was determined that during cultivation macrophages synthesize prostanoids,

Table 1
Spontaneous prostanoid release by peritoneal macrophages

Incubation time (h)	Prostanoid synthesis (ng/10^6 cells)		
	PGE_2	PGF_{2a}	TxB_2
1	nd[a]	nd	nd
2	33±13	31±7	16±2
4	98±12	89±20	24±10
6	85±10	80±13	nd

[a]nd, not detectable.

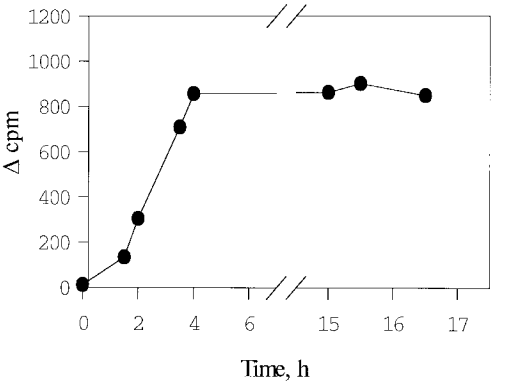

Fig. 1. Time course of [^{3}H]AA metabolite release from mouse peritoneal macrophages. Cells were preincubated at 37°C with [^{3}H]AA for 4 h; excess label was washed off and the cells were incubated at 37°C in DMEM medium with 0.5% BSA. The [^{3}H]AA metabolite content was measured as described in Section 2. Data are presented as the difference (Δ) between the radioactivity in the medium at any time point and the radioactivity at the beginning of the experiment. The results shown are the means of three independent experiments, which varied by less than 7%.

especially PGE_2, PGF_{2a} and TxB_2. The basal prostanoid synthesis was detectable 1 h after medium change, another 3 h later the prostanoid concentrations in the medium were set at a constant level (Table 1). So, non-stimulated peritoneal macrophages are able not only to synthesize prostanoids but also to provide for their constant basal concentration in extracellular medium.

Immune responses are usually the result of close cooperation between lymphocytes and macrophages. To examine the possibility of physiological significance of basal prostanoid synthesis by macrophages in the cellular immune response we selected a cell system from the mouse spleen as it contains both macrophages and lymphocytes. Lymphocytes are known not to synthesize prostanoids but to contain receptors for them [6,7], while macrophages are an important source of PGs. We determined prostanoid synthesis by splenocytes during cultivation and found that non-stimulated cells are able to synthesize prostanoids, particularly PGF_{2a} and PGE_2, and their concentrations in the medium reach a nanomolar basal level ($3.3\pm0.5\times10^{-9}$ M and $3.0\pm0.3\times10^{-9}$ M, respectively) in a few hours. PGE_2 is an effective inhibitor of cell proliferation at 10^{-8}–10^{-7} M [14,15], whereas PGF_{2a} has very little immunomodulatory effect [14,16]. Thus, we further examined only the PGE_2 effect on proliferation.

3.2. PGE_2 influence on the Con A-induced proliferation of splenocytes

The influence of exogenous PGE_2 on the kinetics of Con A-induced proliferation of spleen lymphocytes was studied. It was previously shown that at early incubation times endogenous PGE_2 had not been synthesized. For that reason PGE_2 was added to splenocytes as soon as possible after cell preparation, so its concentrations remained unchanged in the beginning of the experiments. The mitogen and PGE_2 were added simultaneously. The concentrations of 10^{-14}–10^{-7} M of added PGE_2 were both lower and higher than the basal level (10^{-9} M).

We found that added PGE_2 did not change lymphocyte proliferation if its concentration was equal to the basal PGE_2 concentration in the splenocyte cell system (Fig. 2).

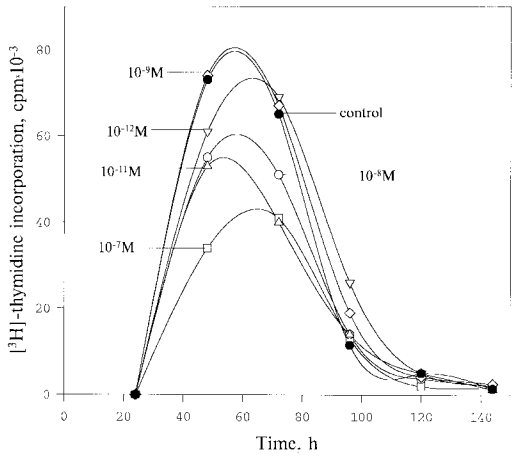

Fig. 2. Time courses of Con A-induced splenocyte proliferation under the influence of different PGE_2 concentrations: 10^{-7} M (□), 10^{-8} M (○), 10^{-9} M (◇), 10^{-11} M (△), 10^{-12} M (▽) and 0 (●, control). The [^{3}H]thymidine incorporation was measured as described in Section 2. Con A (2 μg/ml, final concentration) and PGE_2 were added simultaneously. The results presented here are representative of several separate experiments.

Fig. 2 also shows that 10^{-7} M PGE_2 inhibited the response during the whole time period of lymphocytes proliferation. Surprisingly, PGE_2 at picomolar concentrations possessed the same potent inhibitory activity. The dynamics of the proliferation response was slightly different in different experiments, but the dose-response curves for 48 h and 72 h were the same (Fig. 3). Therefore, at PGE_2 concentrations differing from the basal level (10^{-9} M), both lower and higher, lymphocyte proliferation was suppressed in the same manner.

3.3. *The effect of PGE_2 on lymphocyte proliferation depends on the time of addition*

To demonstrate a direct correlation between the change in the endogenous prostanoid levels and immune cell proliferative activity we examined this phenomenon as a function of the time of PGE_2 addition. In these experiments PGE_2 was added 1 h before mitogen addition or simultaneously with it. The incubation of the cells with PGE_2 prior to mitogen addition led to a considerable increase of the suppressive effect of both high (10^{-8} M) and low (10^{-12} M) concentrations of PGE_2 (Table 2). Our results provide evidence that any change in the basal amount of PGE_2 formed by macrophages may be crucial in the modulation of the cellular immune response.

Table 2
Proliferative lymphocyte index as a function of the time of addition of PGE_2

PGE_2 (M)	Time of PGE_2 addition (h)[a]		Proliferative index[b]	
	−1	0	cpm×10^{-3}	%
0	−	−	65±4	100±6
10^{-8}	−	+	36±2	55±3
	+	−	22±3	34±5
10^{-12}	−	+	58±3	89±5
	+	−	20±5	31±8

[a]PGE_2 was added 1 h before mitogen addition (−1) or simultaneously (0).
[b]Cells were incubated with 2 μg/ml Con A for 72 h, [^{3}H]thymidine was added 24 h before the cells were harvested.

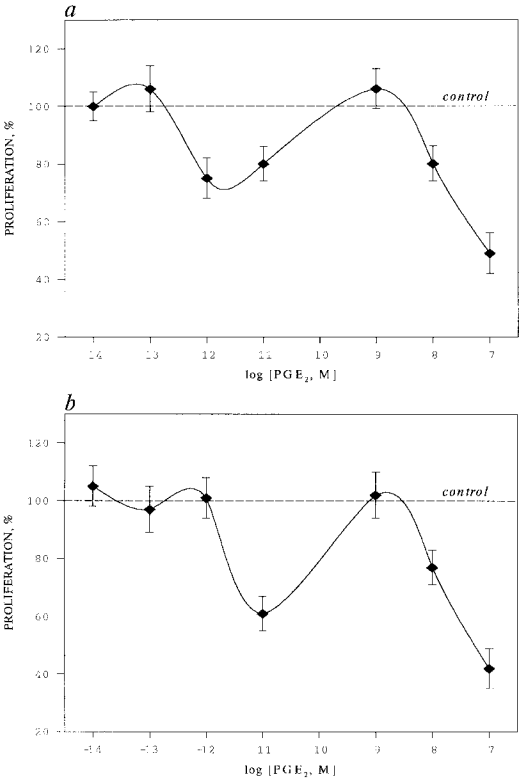

Fig. 3. Dose-response curves for PGE_2 influence on Con A-induced splenocyte proliferation. The proliferation was measured by [^{3}H]thymidine uptake by splenocytes after 48 h (A) or 72 h (B) of incubation. Con A (2 μg/ml, final concentration) and PGE_2 were added simultaneously. Control represents splenocyte Con A-induced proliferation without PGE_2 addition.

Besides, these experiments support the hypothesis that PGE_2 influences the early stages of mitogen action [17].

3.4. *Conclusions*

The results obtained allow us to assume that the macrophage-lymphocyte immune cell system under physiological conditions is 'adapted' to the basal PGE_2 concentration. Generally, an adaptation mechanism explains the effect of exogenous concentrations of a substance in the presence of endogenous concentrations of it. The appropriate model of adaptation is based on the existence of receptors, which when activated conduct fast and slow signals with opposite contributions to the resulting effects [18]. In this case, a change in substance concentration in any direction will cause changes in the system. Such conditions can be reached in the PGE_2 receptor system [6,7,19]. Thus it can be supposed that the level of PGE_2 produced by non-stimulated macrophages has a regulatory role in lymphocyte proliferation. It means that an increase or decrease of the endogenous level of PGE_2 during chronic diseases is capable of changing immune responses. The adaptation of lymphocyte proliferation to the physiological concentration of PGE_2 (10^{-9} M) may be important to clarify the regulation of some immune functions. The increase of prostaglandin synthesis above the normal level is being intensively studied, but the reverse process is neglected. Our results demonstrate that not only the increase but also the decrease of basal PGE_2 concentration, up to the picomolar range, in pathophysiological conditions has regulatory importance for lymphocyte proliferation.

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