Modulation of Interleukin-2-Induced Proliferation and Nonspecific Cytotoxicity of Rat Lymphocytes by Phorbol Myristate Acetate and Staurosporine

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It is shown that a 19-h pretreatment of rat splenocytes with 1 μM phorbol 12-myristate 13-acetate followed by a 42-h incubation with human recombinant interleukin-2 inhibits nonspecific cytotoxicity of these cells toward the target YAC-1 cells. By contrast, proliferation of splenocytes and thymocytes incubated under the same conditions increases considerably. The inhibitor of protein kinase C staurosporine (0.1 μM) significantly decreases nonspecific cytotoxicity of splenocytes after a 20-min incubation, while in a dose of 0.01 μM it stimulates lytic activity of splenocytes and thymocytes following a 3-day incubation with interleukin-2 in the presence of the inhibitor. Cell proliferation under these conditions is markedly decreased.

Key Words: *splenocytes; thymocytes; phorbol myristate acetate; staurosporine*

Interleukin-2 (IL-2) is known to stimulate or induce *de novo* a nonrestricted by the major histocompatibility complex antigens cytotoxicity (NCT) of lymphocytes in peripheral lymphoid organs and blood, which is aimed at eradicating tumor or virus-infected cells [5]. A similar activity was demonstrated for other growth factors, specifically, IL-4 and IL-7 [13]. Depending on the incubation time, stimulation or *de novo* generation of NCT of cultured cells in the presence of these interleukins is accompanied by cell proliferation. Regulation of two functional activities of the receptor for IL-2 (induction of proliferation and stimulation of NCT) may be provided by different second messengers (for example, tyrosine protein kinase and/or GTP-binding protein) coupled to the intercellular domain of the IL-2 receptor.

Modulation of normal NCT activity was documented for effector cells treated with the agents stimulating Ca^{2+},phospholipid-dependent protein kinase C (PKC) [11]. Since IL-2 was shown to stimulate PKC in a variety of experimental systems [7], it can be suggested that it stimulates (induces *de novo*) NCT (but not cell proliferation) via activation of PKC [1]. Bearing in mind that NCT is inhibited by chronic treatment of cells with high doses of phorbol 12-myristate 13-acetate (PMA) [11], it was interesting to examine the effects of this treatment on the inhibition of PKC with staurosporine [14] and on its stimulation with IL-2.

MATERIALS AND METHODS

Male Wistar rats weighing 170-220 g were used. Thymocyte and splenocyte suspensions were prepared by pressing fragments of the thymus and spleen through steel mesh in medium 199. Erythrocytes in the splenocyte suspension were lysed with distilled water (30 sec), after which the suspension was twice washed with medium 199. More than 95% of cells in the suspension were viable (trypan blue exclusion test). The cells were cultured and tested for NCT activity in complete RPMI-1640 medium containing 10% fetal calf serum (N. F. Gamaleya

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Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol (Ferak), 100 U/ml penicillin, and 100 μg/ml streptomycin. The NCT test (14-h incubation) was performed as described [3]. Murine YAC-1 line sensitive to natural killer cells was used as target cells. YAC-1 cells were radiolabeled by a 2-h incubation with 10 μCi/ml ^{3}H-uridine. The effector:target ratios were 25:1, 12:1, 6:1, and 3:1. Residual radioactivity after cell transfer to filters was measured in a Beta-2 liquid scintillation counter. The cytotoxicity index was calculated from the following formula: [1-(cpm in experiment)/(cpm in control)]×100%. The target cells incubated under the same conditions without splenocytes served as the control. Round-bottom 96-well microplates (Lenmedpolimer) were used for the NCT test and DNA synthesis assay.

The synthesis of DNA was assessed in splenocytes grown for 72 h in complete medium 199. Cell concentration was 2×10^5/0.2 ml. ^{3}H-Methylthymidine (1 μCi) was added 24 h before the end of incubation. Cells were transferred onto capron membranes using a semiautomatic harvester, and incorporated radiation was measured in the Beta-2 counter.

Cells were preincubated with PMA (1 μM) in complete medium for 19 h at 37°C in humidified atmosphere with 5% CO_2. An equal amount of the solvent was added to the control cultures to a final concentration of 0.06%. After incubation, the cells were twice washed with medium 199.

Staurosporine, PMA, concanavalin A were from Sigma (USA), human recombinant IL-2 (hrIL-2) was from Biogen (Russia), ^{3}H-uridine and ^{3}H-methylthymidine were from Izotop (Russia). Other reagents were Russian-manufactured of chemically pure grade.

The significance of differences was evaluated using Student's *t* test.

RESULTS

Previously, we showed that incubation of rat splenocytes with hrIL-2 for 42 h (up to 3 days) increases nonspecific lysis of target cells (YAC-1) in a 14-h test with the release of ^{3}H-uridine-labeled RNA [6]. Therefore, 42 h was assumed as a minimal incubation time required to enhance NCT of these cells in response to hrIL-2.

Preincubation of splenocytes with PMA for 19 h significantly decreased NCT during subsequent 42-h incubation with hrIL-2 (Fig. 1). This may be due to lowered lytic activity but not to decreased viability of these cells, since the number of splenocytes including trypan blue after PMA treatment was never higher than 10-15%. It was reported that NCT activity markedly decreases after a 48-h incubation of human large granular lymphocytes with 1.67 μM PMA. Since under our experimental conditions hrIL-2 cannot restore normal NCT activity of PMA-treated splenocytes during a 42-h incubation, the possibility that this time is insufficient for the restoration of exhausted PKC pool cannot be ruled out. By contrast, splenocyte proliferation increased considerably (Fig. 2), which agrees with the

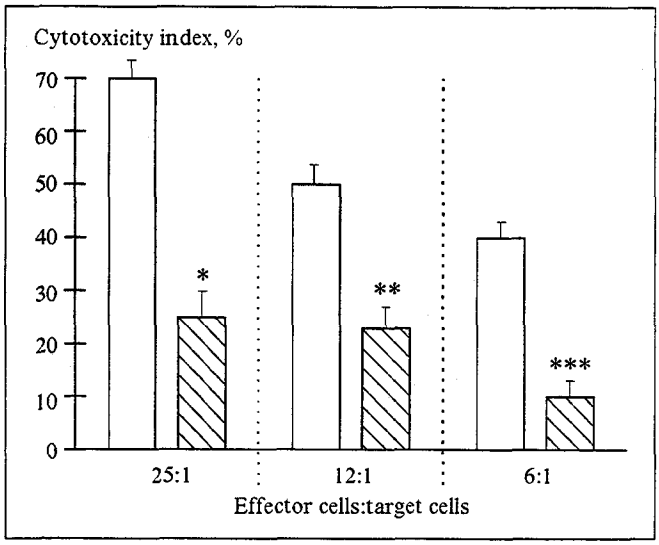

Fig. 1. Effect of preincubation with phorbol 12-myristate 13-acetate on nonspecific cytotoxicity of splenocytes incubated with human recombinant IL-2 (200 units/ml). White bars: control splenocytes; shaded bars: preincubated splenocytes. *p<0.001; **p<0.02; ***p<0.01 compared with the control.

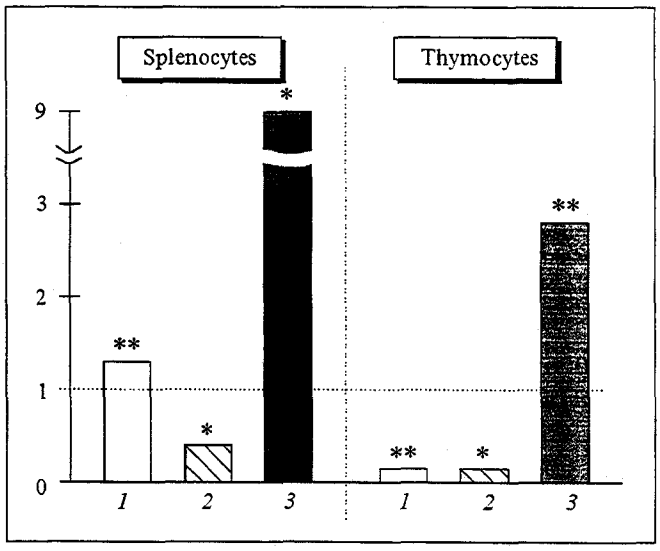

Fig. 2. Effect of preincubation with phorbol 12-myristate 13-acetate on spontaneous and induced proliferation of splenocytes. *1*) cultures without stimulators; *2*) concanavalin A (5 μg/ml); *3*) human recombinant interleukin-2 (200 units/ml). Ordinate: ratio (cpm) between preincubated to control samples with and without the simulators. *p<0.001; **p<0.02.

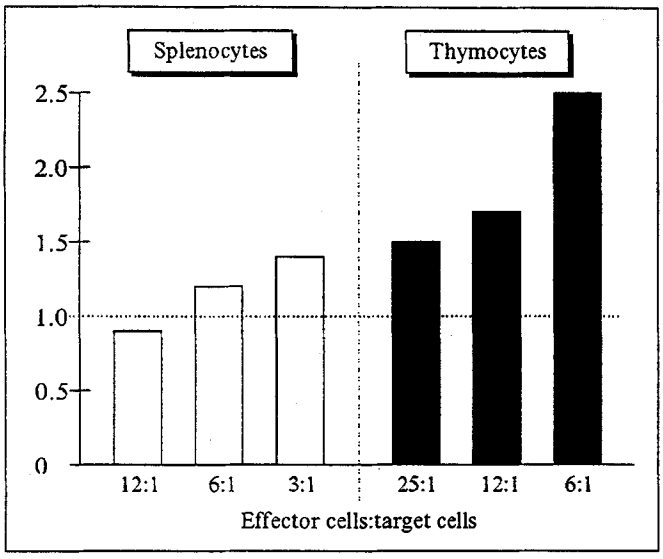

Fig. 3. Effect of staurosporine (0.01 μM) of IL-2-induced nonspecific cytotoxic activity of rat lymphocytes. Ordinate: the ratio between the cytotoxicity index in samples with and without staurosporine.

observation of LeGrue [8]. This effect may be due to "switching off" PKC that mediates the negative feedback loop [10]. Pretreatment with 1 μM PMA induces *de novo* expression of the α-subunit of IL-2 receptor, which may enhance cell proliferation in response to hrIL-2. The incorporation of ^{3}H-thymidine by PMA-treated cells in response to concanavalin A decreased considerably, which may be explained by the fact that concanavalin A cannot activate lymphocytes preincubated with high doses of PMA.

Since prolonged (19 h and longer) incubation of splenocytes with high doses of PMA *in vitro* inhibits their NCT probably due to exhaustion of PKC activity, a problem arises concerning the specificity of PMA action and potential uncontrolled contribution of the *de novo* synthesized PKC molecules to the effect of PMA. In an attempt to solve this problem we reproduced the effects of high PMA doses with staurosporine. Preliminary studies showed that a 20-min preincubation of splenocytes with 0.1 μM staurosporine significantly reduces lysis of YAC-1 by 9-17% (at the effector:target ratios 12:1 and 25:1). Since staurosporine cannot activate PKC (in contrast to PMA), i.e., at any concentration it is a true inhibitor, it was added to the culture medium in a concentration of 0.01 μM. At this concentration staurosporine did not block completely cell proliferation in response to hrIL-2 or concanavalin A (Table 1). An increase in the inhibitor concentration to 0.033 μM and higher abolished the incorporation of ^{3}H-thymidine in rat splenocytes. At the same time, staurosporine (0.01 μM) did not reduce significantly the NCT activity of these cells and even increased it at the effector:target ratios of 6:1 and 3:1 in the presence of hrIL-2 under the same incubation conditions (Fig. 3). It should be noted that the activity of partially purified PKC from rat brain is inhibited by 50% in the presence of 0.003 μM staurosporine [14].

In this study we used the total fraction of nuclear splenocytes containing adherent (A) cells. It was shown that the presence of the effectors of A cells reduces the IL-2 dependent *de novo* generation of NCT toward the natural killer (NK)-resistant Raji cells and potentiates NCT toward the NK-sensitive K562 cells [2]. Proceeding from the fact that any other subpopulation of lymphocytes may modulate NCT, it can be suggested that the effects of PMA and staurosporine result from indirect influence of these agents on this "intermediate" lymphocyte subpopulation regulating the activity of NK. It can be hypothesized that if NCT is induced by IL-2 in lytically inactive cells (thymocytes), the population of which in mice [4] and humans [9] contains no detectable amounts of mature $CD3^-$ NK of the corresponding phenotype, and the mechanisms of activation and NCT activity of $CD3^+$ T cells and $CD3^-$ NK are similar, PKC inhibitors should elicit their effect on NCT and proliferation of IL-2-stimulated cells.

TABLE 1. Modulation of Spontaneous and Induced Proliferation of Rat Lymphocytes in the Presence of Staurosporine

Cells	Stimulator	Staurosporine concentration, μM		
		0	0.01	0.033
Splenocytes	—	4 827±1 048	324±16 (7)	216±1 (4)
	Concanavalin A, 5 μg/ml	16 841±908	2 780±404 (17)	225±6 (1)
	hrIL-2, 200 units/ml	5 374±360	1 279±123 (24)	228±7 (4)
Thymocytes	—	742±100	534±25 (72)	n.d.
	Concanavalin A, 5 μg/ml	35 262±748	15 494±308 (44)	n.d.
	hrIL-2, 200 units/ml	5 977±667	2 888±500 (48)	n.d.

Note. n.d. = not determined. The changes (%) are given in parentheses.

The effects of staurosporine on proliferation and NCT of hrIL-2-stimulated thymocytes are similar to its effect on splenocytes (Table 1, Fig. 3). Since the original NCT activity of thymocytes is very low (5-7% and lower) and does not increase after a 42-h incubation with hrIL-2, we present the data only on the effect of PMA on proliferation of these cells (Fig. 2). There was no principal difference between PMA-activated proliferation of splenocytes and thymocytes. However, the studied PKC inhibitors exert absolutely different effects (inhibition/activation). This may be due to the fact that staurosporine is not a specific PKC inhibitor: under certain conditions it inhibits tyrosine protein kinase [12]. The role of heterogeneity of the PKC family cannot be ruled out [10].

Thus, dissociation of proliferative and NCT-inducing activity of hrIL-2 has been observed in experiments with PKC inhibitors. The NCT activity significantly decreases after a 20-min preincubation of splenocytes with 0.1 μM staurosporine. A tendency toward an increase in the nonspecific lysis of YAC-1 cells in hrIL-2 stimulated cultures of splenocytes and thymocytes in the presence of 0.01 μM staurosporine may arise from the effect of the inhibitor on other signal systems that mask PKC inhibition.

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