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ORIGINAL ARTICLE

Joan M. Cook-Mills · Margalit B. Mokyr
Rhonna L. Cohen · Robert L. Perlman · Donald A. Chambers

Neurotransmitter suppression of the in vitro generation of a cytotoxic T lymphocyte response against the syngeneic MOPC-315 plasmacytoma

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Abstract We have previously shown that, as a consequence of low-dose melphalan (L-phenylalanine mustard (L-PAM) therapy, the hitherto immunosuppressed spleen cells from BALB/c mice bearing a large MOPC-315 tumor (in contrast to spleen cells from normal mice) acquire the ability to generate a greatly enhanced anti-MOPC-315 cytotoxic T lymphocyte (CTL) response upon in vitro stimulation with MOPC-315 tumor cells. Here we show that the catecholamines norepinephrine, epinephrine, and isoproterenol suppressed the in vitro generation of anti-MOPC-315 cytotoxicity by spleen cells from mice that had just completed the eradication of a large MOPC-315 tumor following low-dose L-PAM therapy (L-PAM TuB spleen cells), as well as by spleen cells from normal mice. In contrast to the marked suppression obtained with catecholamines, the cholinergic agonist carbachol had no effect on the in vitro generation of splenic anti-MOPC-315 cytotoxicity. The inhibitory effect of the catecholamines was "mimicked" by the membrane-penetrating analog of cAMP, dibutyryl-cAMP, and by cholera toxin at concentrations that stimulate the endogenous production of cAMP. The β-adrenergic receptor antagonist propranolol did not block norepinephrine-induced inhibition of the generation of anti-MOPC-315 cytotoxicity by either normal or L-PAM TuB spleen cells. Since the curative effectiveness of low-dose L-PAM therapy for MOPC-315 tumor bearers requires the participation of $CD8^+$ T cells that exploit a CTL response in tumor eradication, it is conceivable that norepinephrine may reduce the therapeutic outcome of low-dose chemotherapy by inhibiting the acquisition of CTL activity.

Key words Catecholamine · CTL · Low-dose chemotherapy

J. M. Cook-Mills · R. L. Cohen · D. A. Chambers
Center for Molecular Biology of Oral Diseases,
University of Illinois at Chicago, USA

M. B. Mokyr · D. A. Chambers
Department of Biochemistry, University of Illinois at Chicago, USA

D. A. Chambers (✉)
Department of Biochemistry,
University of Illinois College of Medicine,
1853 West Polk Street (M/C 536) Chicago, IL 60612, USA

R. L. Perlman
Departments of Pediatrics and of
Pharmacological and Physiological Sciences,
and
The Joseph P. Kennedy Jr. Mental Retardation Research Center,
The University of Chicago, USA

Introduction

Extensive studies carried out in recent years have yielded new insights into interactions between the nervous system and the immune system (for review see [5]). For example, it has been shown that lymphoid organs (e.g., spleen) are innervated by the sympathetic nervous system and that synaptic-like junctions bathed by neuromodulators occur between neurons and lymphocytes [11, 12]. In addition, lymphocytes have receptors that can respond to a variety of neurotransmitters, including the classical sympathetic neurotransmitter norepinephrine [2, 13, 14, 19, 20, 27, 37].

Studies of the immunoregulatory properties of neurotransmitters yielded mixed results. They revealed that catecholamines can inhibit proliferation of B lymphocytes and T lymphocytes in vitro [8, 14, 34]. In contrast, the potential inhibitory activity of catecholamines in vivo was deduced from studies demonstrating that sympathetic denervation of the spleen enhances antibody production [2, 12, 20]. However, the effects of catecholamines on lymphocytes are not

invariably immunosuppressive. In fact, in an allogeneic system, catecholamines have been reported to enhance the in vitro generation of cytotoxic T lymphocytes (CTL) [12, 15]. Since catecholamines have been shown to increase some lymphocyte responses while decreasing others, it is difficult to predict the effects of neurotransmitters on the generation of CTL activity against syngeneic tumor cells.

The current study was undertaken to examine the effect(s) of neurotransmitters on the generation of an antitumor CTL response in light of the growing appreciation of both the importance of CTL in tumor eradication [10, 16, 22, 33] and the immunoregulatory properties of neuromodulators in immune regulation. As a model in which CTL participate in tumor eradication in vivo, we selected the MOPC-315 plasmacytoma. In this system, $CD8^+$ T cells acquire a potent CTL response as a consequence of low-dose melphalan (L phenylalanine mustard, L-PAM) therapy of mice bearing large (more than 20 mm) subcutaneous (s.c.) tumors with extensive metastases (L-PAM TuB mice). The resultant $CD8^+$ T-cell-dependent CTL immunity is responsible for the eradication of the large tumor burden, which is not eradicated by the direct antitumor effects of the low dose of drug [22]. Although the exact magnitude of tumor burden eradicated by the $CD8^+$ T cells under these conditions has not yet been determined, it is most likely large since, in the absence of the contribution of $CD8^+$ T cells, the tumoricidal/tumoristatic activities of the low dose of L-PAM are insufficient to eradicate a barely palpable tumor [22].

In assessing the effect of neurotransmitters on the generation of anti-MOPC-315 CTL activity, we took advantage of the fact that the exact conditions for the in vitro generation of anti-MOPC-315 CTL response are well established [21]. Accordingly, we studied the effects of catecholamines and other neuromodulators on the in vitro generation of an anti-MOPC-315 CTL response by spleen cells from L-PAM TuB BALB/c mice as well as by spleen cells from normal BALB/c mice.

Materials and methods

Tumor

We have employed primarily the weakly immunogenic [26] MOPC-315 plasmacytoma, which was maintained in vivo as previously described [21] as a s.c. tumor in female BALB/c mice (Charles Rivers Breeding Laboratories, Wilmington, Mass.). Routinely the mice were injected s.c. with 1×10^6 viable MOPC-315 tumor cells, a dose that is about 300 times the minimal lethal tumor dose, and which kills the mice in approximately 16 days.

Chemotherapy

A fresh stock solution of 10 mg/ml L-PAM (Burroughs Wellcome Co., Triangle Park, N.C.) was prepared as previously described [1], and was further diluted with Dulbecco's phosphate-buffered saline, pH 7.2 (Gibco, Grand Island Biological Co., Grand Island, N.Y.) to the desired concentration. A dose of 2.5 mg L-PAM/kg body weight (mg/kg) was administered intraperitoneally to mice bearing a large (at least 20 mm) MOPC-315 tumor, which resulted from the s.c. inoculation of 1×10^6 MOPC-315 tumor cells 10 days earlier (L-PAM TuB mice). This dose of L-PAM is curative for approximately 90% of mice bearing a large MOPC-315 tumor and leads to the complete regression of the s.c. tumor nodule within 8–10 days after L-PAM administration [1, 22, 33]. The curative effectiveness of 2.5 mg/kg L-PAM depends on the participation of $CD8^+$ T cells in tumor eradication.

Spleen cell suspensions

Single-cell suspensions were prepared from the spleens of normal BALB/c mice or BALB/c L-PAM TuB mice (L-PAM TuB spleen cells) that had been treated 10–15 days earlier with low-dose L-PAM when the mice bore a 20-mm subcutaneous tumor (i.e. mice that just completed tumor eradication). The single-cell suspensions were prepared by mincing normal spleens and gently pressing the cells through a sterile Nytex nylon mesh (Tetko Inc., Elmsford, N.Y.) with a sterile stainless-steel lab spoon (American Scientific Products, McGaw Park, Ill.). In any individual experiment, pooled spleen cells from at least five mice were used.

Chemicals

All neurotransmitters and related compounds used in these studies were purchased from Sigma, St. Louis, Mo.

In vitro stimulation for the generation of CTL

Normal or L-PAM TuB spleen cells were stimulated in vitro with MOPC-315 tumor cells according to the method we have previously described [21]. Briefly, 40×10^6 normal spleen cells were admixed with 1.33×10^6 mitomycin-C-treated (50 μg/ml for 30 min) MOPC-315 tumor cells in 20 ml medium consisting of RPMI-1640 medium supplemented with 1% nonessential amino acids (Gibco, Grand Island, N.Y.), 2 mM glutamine, 50 μM 2-mercaptoethanol (Sigma, St. Louis, Mo.), 50 U/ml penicillin, 50 μg/ml streptomycin (Sigma) and 5% heat-inactivated fetal bovine serum (Gibco). Agonists were added at the time of culture initiation unless otherwise stated. At the concentrations used, the solvent for the neuromodulators, HCl (at a final concentration of 0.1 mM or 10 μM in buffered medium), had no effect on the generation of anti-MOPC-315 cytotoxicity. The cultures were incubated at 37° C in 5% CO_2/air for 5 days unless otherwise stated, since the generation of the anti-MOPC-315 CTL response by spleen cells from normal BALB/c mice peaks between days 4 and 6 [21].

Cytolytic assay

The cytolytic activity of the cultured spleen cells was determined by the ^{51}Cr-release assay as previously described [21]. Briefly, the cultured spleen cells were incubated for 3.5 h with 5×10^4 ^{51}Cr-labelled MOPC-315 target cells at effector-to-target cell (E/T) ratios of 200:1, 100:1, 50:1, and 25:1. The results are expressed as $LU_{20}/10^7$ effector cells with 1 LU_{20} defined as the number of spleen cells required to lyse 20% of the target cells (Clinical Immunology Services, Program Resources Inc., FCRF/NCI, Frederick, Md.). We have observed, as have others [3], that the level of antitumor cytotoxicity generated by spleen cells from individual mice

stimulated in vitro with tumor cells under the same culture conditions may vary substantially from one experiment to another. However, the pattern of results remained consistent. The results of two representative experiments are therefore presented in each figure.

Adenylyl cyclase activity in permeabilized spleen cells

Spleen cells were saponin-permeabilized by the method of Rasenick and Kaplan [25] and assayed for adenylyl cyclase activity. Briefly, 1.5×10^8 spleen lymphocytes were suspended in 3 ml saponin solution (140 mM potassium glutamate, pH 6.8, 2 mM ATP, 100 μg/ml saponin). After 160 s, 40 ml 140 mM potassium glutamate was added to the cells followed by three washes with Hanks buffer (Gibco). Adenylyl cyclase activity was measured in a final assay volume of 0.4 ml. The reaction solution contained 0.5 mM ATP, 1 mM $MgCl_2$, 0.5 mM isobutylmethylxanthine $(2–3) \times 10^8$ cpm per tube [α-^{32}P]ATP (610 Ci/mmol, ICN, Lisle, Ill.) in Hanks buffer. Propranolol (10 μM–10 mM) was added to the reaction solution containing saponin-permeabilized spleen cells (50–100 μg total cell protein/tube). After 5 min at room temperature, isoproterenol (50 μM) was added to activate adenylyl cyclase. The tubes were incubated for 15 min at 32° C. Reactions were terminated by boiling for 5 min. The [^{32}P]cAMP formed during the assay was isolated by sequential chromatography on Dowex and alumina columns as described by Salomon [28].

Statistical analysis

To determine if the level of anti-MOPC-315 cytotoxicity exhibited by an experimental group is significantly different from that exhibited by the control group, we have used a completely random analysis of variance followed by Dunnett's *t*-test [30]. A *P* value below 0.05 was considered significant.

Results

Effects of catecholamine agonists on the in vitro generation of anti-MOPC-315 cytotoxicity by spleen cells from normal or L-PAM TuB mice

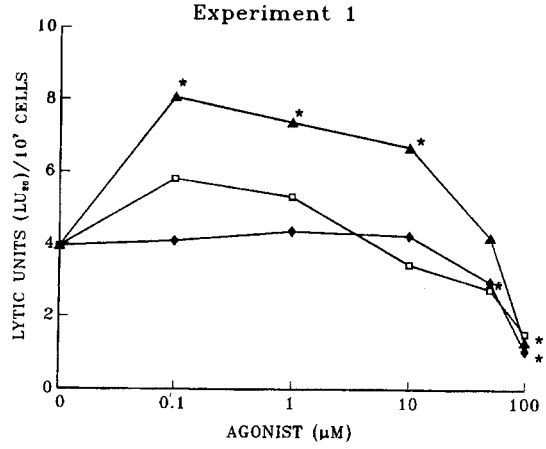
Experiments were performed to determine if addition of the catecholamines norepinephrine, epinephrine, or isoproterenol at the initiation of a 5-day in vitro stimulation culture of normal or L-PAM TuB spleen cells and mitomycin-C-treated MOPC-315 tumor cells would affect the generation of antitumor cytotoxicity. Spleen cells from both sources were also cultured with mitomycin-C-treated MOPC-315 cells in the absence of catecholamines. The generation of anti-MOPC-315 cytotoxicity by normal spleen cells was significantly augmented when they were cultured in the presence of norepinephrine at low concentrations (0.1–1 μM), but not epinephrine or isoproterenol (Fig. 1). When the concentration of catecholamines added to the in vitro stimulation cultures was increased to 50–100 μM, all of the catecholamines inhibited the generation of anti-MOPC-315 cytotoxicity by normal spleen cells (Fig. 1) and L-PAM TuB spleen cells (Fig. 2a). To illustrate the reproducibility of the effect of catecholamines on the generation of anti-MOPC-315 cytotoxicity, we provide in Fig. 2b the compiled data from all (eight) experiments in which norepinephrine was added to L-PAM TuB spleen cell cultures. From these data it is apparent that, as the concentration of norepinephrine increased from 50 μM to 100 μM, the magnitude of antitumor cytotoxicity generated by the L-PAM TuB spleen cells decreased. Since such an effect could be a result of catecholamine-induced cytotoxicity, we determined if the decrease in the level of antitumor cytotoxicity generated in the presence of increasing concentrations of catecholamines was due to a gradual decrease in the viability of the spleen cells. Catecholamines at concentrations of 50–100 μM did not reduce spleen cell viability on day 2 of the culture or reduce the total number of viable lymphocytes harvested on day 5 after culture initiation (Table 1).

Effects of cholinergic agonists on the generation of anti-MOPC-315 cytotoxicity

Experiments were performed to investigate if cholinergic agonists affect the generation of anti-MOPC-315 cytotoxicity in ways similar to norepinephrine. In these studies we have used normal spleen cells, because the enhancing effect of the lower concentrations of norepinephrine on the generation of anti-MOPC-315 cytotoxicity could be seen only with normal spleen cells, whereas the suppressive effect of the higher concentrations of drug was evident with both normal and L-PAM TuB spleen cells. Accordingly, carbachol (a cholinergic agonist) was added at the initiation of a 5-day in vitro stimulation culture. At low concentrations, carbachol increased the generation of antitumor cytotoxicity (Fig. 3). However, in contrast to the catecholamines (Figs. 1, 2), at the higher concentrations carbachol did not inhibit the generation of antitumor cytotoxic activity but actually enhanced it.

Effect of cyclic AMP on the generation of antitumor cytotoxicity

Since previous studies suggested that cAMP can be a second messenger for catecholamine-induced inhibition of T cell proliferation [4, 5, 14, 34], experiments were performed to determine if cAMP, at concentrations shown to inhibit lymphocyte DNA synthesis [6], would also inhibit the generation of anti-MOPC-315 cytotoxicity. For this purpose, we used nontoxic concentrations of dibutyryl-cAMP (a membrane-penetrating analog of cAMP) and cholera toxin (which stimulates endogenous production of cAMP). Dibutyryl-cAMP and cholera toxin did indeed inhibit the generation of anti-MOPC-315 cytotoxicity by spleen cells from normal (Fig. 4) as well as from L-PAM TuB (Fig. 5) mice.



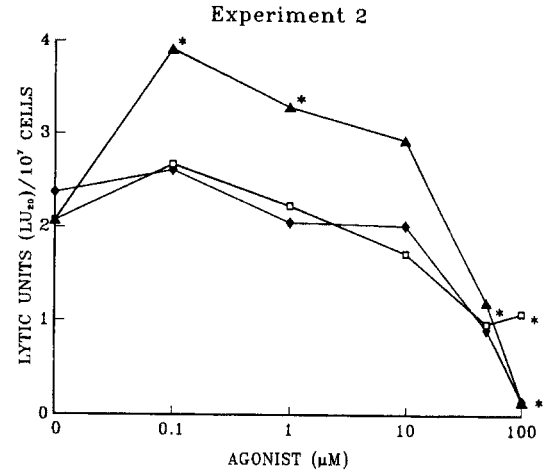
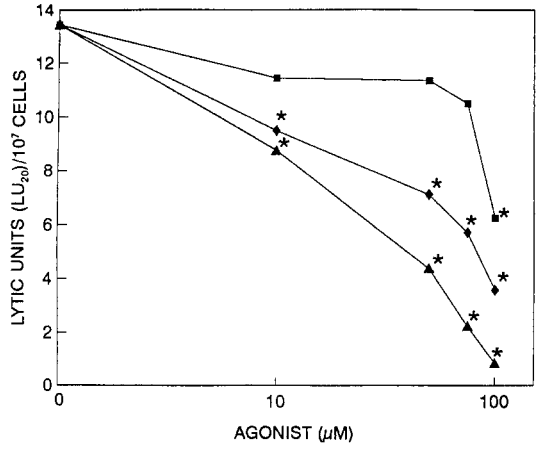


Fig. 1 Effect of catecholamines on the in vitro generation of anti-MOPC-315 cytotoxicity by normal BALB/c spleen cells. Norepinephrine (▲), epinephrine (□) or isoproterenol (◆) was added to the culture mixture of normal BALB/c spleen cells and mitomycin-C-treated syngeneic MOPC-315 tumor cells. The anti-MOPC-315 lytic activity was determined 5 days later by ^{51}Cr-release assay. The results are presented as $LU_{20}/10^7$ effector cells calculated from the mean percentage specific ^{51}Cr release. *Statistical significance ($P < 0.05$) relative to lytic activity exhibited by spleen cells stimulated with MOPC-315 tumor cells in the presence of the HCl solvent which, by itself, had no effect on the level of antitumor cytotoxicity generated



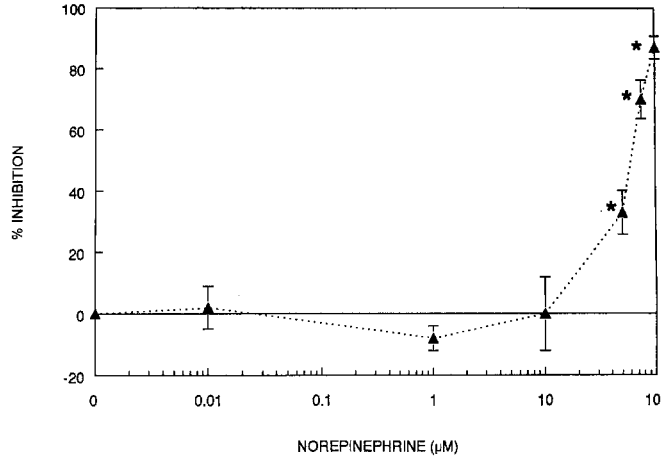


Fig. 2 A Effect of catecholamines on the in vitro generation of anti-MOPC-315 cytotoxicity by spleen cells from melphalan(L-PAM)-treated MOPC-315-tumor bearing mice. Norepinephrine (▲), epinephrine (□) or isoproterenol (◆) was added to the culture mixture of L-PAM-treated tumor-bearer BALB/c spleen cells and mitomycin-C-treated syngeneic MOPC-315 tumor cells. The anti-MOPC-315 lytic activity was determined 5 days later by ^{51}Cr-release assay. The results are presented as $LU_{20}/10^7$ effector cells calculated from mean percentage specific ^{51}Cr release. *Statistical significance ($P < 0.05$) relative to lytic activity exhibited by spleen cells stimulated with MOPC-315 tumor cells in the presence of the HCl solvent. **B** Effect of norepinephrine on the in vitro generation of anti-MOPC-315 cytotoxicity by spleen cells from L-PAM-treated MOPC-315-tumor-bearing mice: compiled data of eight experiments. Norepinephrine (▲) was added to the culture mixture of L-PAM-treated tumor-bearer BALB/c spleen cells and mitomycin-C-treated syngeneic MOPC-315 tumor cells. The anti-MOPC-315 lytic activity was determined 5 days later by ^{51}Cr-release assay

Effect of the β-adrenergic receptor antagonist propranolol on norepinephrine-mediated inhibition of the generation of antitumor cytotoxicity

Since cytotoxic T lymphocytes have β_2-adrenergic receptors coupled to adenylyl cyclase (reviewed in [27]), experiments were performed to determine whether the norepinephrine inhibition of the generation of anti-MOPC-315 cytotoxicity could be affected by β-adrenergic antagonists. For this purpose, the ability of propranolol, a β-adrenergic receptor antagonist, to block the inhibitory effect of the catecholamine norepinephrine on the generation of anti-MOPC-315 cytotoxicity was studied. Normal (Fig. 6) or L-PAM TuB (Fig. 7) spleen cells were incubated with 0.1–10 μM propranolol, which by itself did not affect the generation of antitumor cytotoxicity. After 30 min,

Table 1 Effects of neurotransmitters on viable cell recovery. Spleen cells (40×10^6) were cultured in the presence or absence of neurotransmitters or the solvent, HCl. After 5 days the cells were washed twice and the number of viable cells determined by trypan blue exclusion. Data are from the representative experiment illustrated in Fig. 1. Viable cell recovery of spleen cells incubated in the absence of MOPC-315 cells or neurotransmitters was 15.7×10^6 cells

Spleen cells stimulated with MOPC-315 tumor cells in the presence of:	$10^{-6} \times$ Viable cells recovered
Medium only	27.2
HCl, 100 µl	23.7
HCl, 50 µl	19.4
Norepinephrine, 100 µM	20.4
Norepinephrine, 1 µM	24.8
Epinephrine, 100 µM	30.3
Epinephrine, 1 µM	21.1
Isoproterenol, 100 µM	24.9
Isoproterenol, 1 µM	20.8

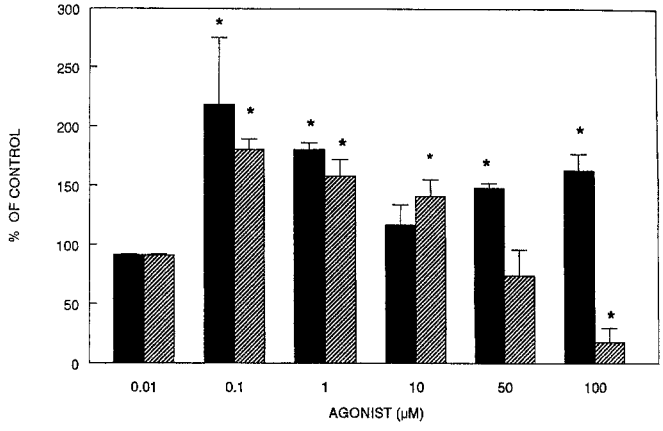


Fig. 3 Effect of carbachol and norepinephrine on the generation of anti-MOPC-315 cytotoxicity by normal spleen cells. Carbachol (■) or norepinephrine (▨) was added to the culture mixture of normal BALB/c spleen cells and the anti-MOPC-315 lytic activity was determined 5 days later by ^{51}Cr-release assay. The results of two experiments are presented as the percentage of the HCl control. *Statistical significance ($P < 0.01$)

norepinephrine and mitomycin-C-treated MOPC-315 cells were added to the spleen cells. After 5 days in culture, cytotoxic activity was measured. Propranolol at a concentration of 0.1–10 µM did not block the norepinephrine-induced (50 µM and 100 µM) inhibition of the generation of anti-MOPC-315 cytotoxicity by spleen cells from either normal or L-PAM TuB mice. Higher concentrations of propranolol were not used since they were toxic. In a parallel experiment, propranolol, at a concentration of 1 µM, blocked isoproterenol stimulation of adenylyl cyclase activity in cells from the same preparation of normal spleen cells (Fig. 8) revealing that a classic β-adrenergic response could be blocked by a β-adrenergic antagonist in this system. Thus, propranolol, at a concentration that blocked isoproterenol-mediated stimulation of adenylyl cyclase via the β-adrenergic receptor, was unable to block the norepinephrine-mediated inhibition of the generation of anti-MOPC-315 cytotoxicity.

Discussion

The results presented here illustrate that catecholamines can suppress the in vitro generation of antitumor cytotoxicity by spleen cells from normal as well as from L-PAM TuB mice. This inhibitory activity of catecholamines was "mimicked" by dibutyryl-cAMP and cholera toxin. However, propranolol, at a concentration that blocked the isoproterenol-mediated stimulation of adenylyl cyclase via β-adrenergic receptors, did not block the norepinephrine-mediated inhibition of the generation of anti-MOPC-315 cytotoxicity. Thus, it is possible that norepinephrine mediated its inhibitory activity on the generation of anti-MOPC-315 cytotoxicity through non-β-adrenergic receptors coupled to adenylyl cyclase [7, 24, 31, 35], or through an altered β-adrenergic receptor.

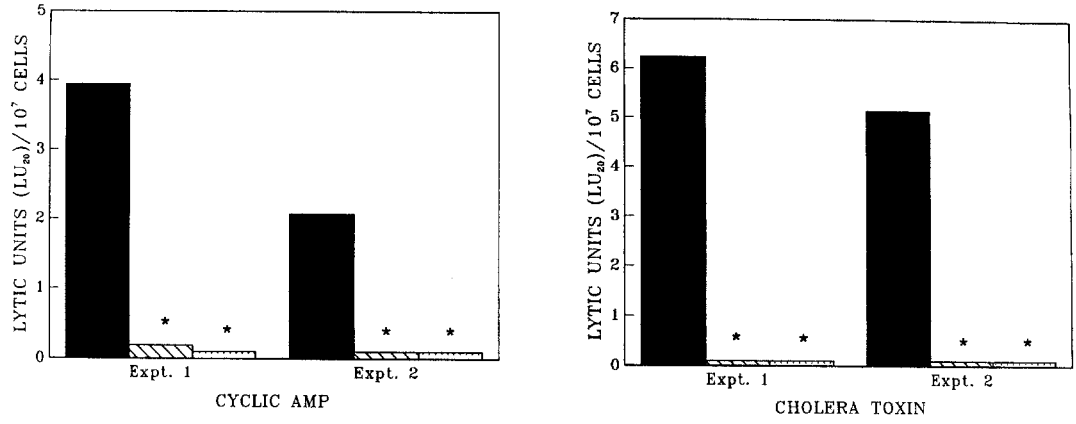


Fig. 4 Effect of dibutyryl-cAMP on the generation of anti-MOPC-315 cytotoxicity by normal BALB/c spleen cells. Dibutyryl-cAMP at a concentration of 100 µM (▨) or 500 µM (⁞⁞) and cholera toxin at a concentration of 2.5 nM (▨) or 1 nM (⁞⁞) and solvent controls (■) were added to the culture mixture of normal BALB/c spleen cells and mitomycin-C-treated syngeneic MOPC-315 tumor cells. The anti-MOPC-315 lytic activity was determined 5-days later by the ^{51}Cr-release assay. *Statistical significance ($P < 0.05$) relative to lytic activity of spleen cells incubated with mitomycin-C-treated MOPC-315 tumor cells

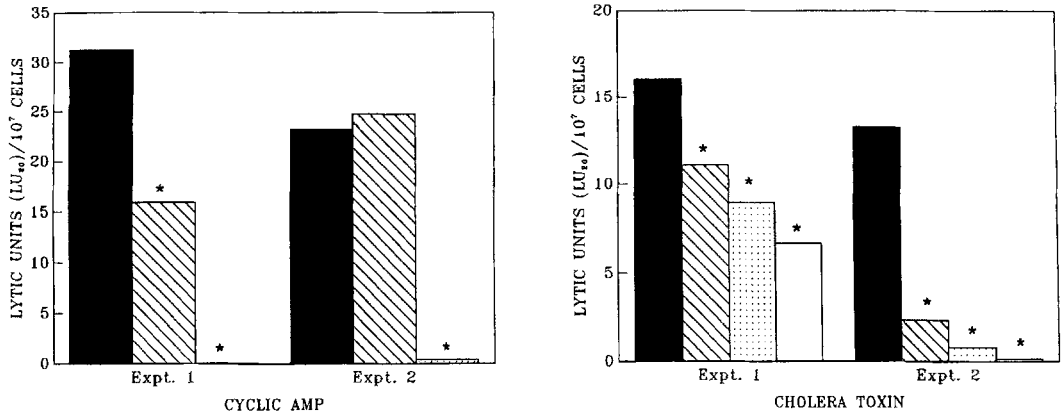


Fig. 5 Effect of dibutyryl-cAMP and cholera toxin on the generation of anti-MOPC-315 cytotoxicity by spleen cells from L-PAM-treated tumor-bearing mice (L-PAM TuB). Dibutyryl-cAMP at a concentration of 100 μM (▨) or 500 μM (⋯) and cholera toxin at a concentration of 5 nM (▨), 2.5 nM (⋯) or 1 nM (□) and solvent controls (■) was added to the culture mixture of L-PAM TuB spleen cells and mitomycin-C-treated syngeneic MOPC-315 tumor cells. The anti-MOPC-315 lytic activity was determined 5 days later by the ^{51}Cr-release assay. *Statistical significance ($P < 0.05$) relative to lytic activity of spleen cells incubated with mitomycin-C-treated MOPC-315 tumor cells

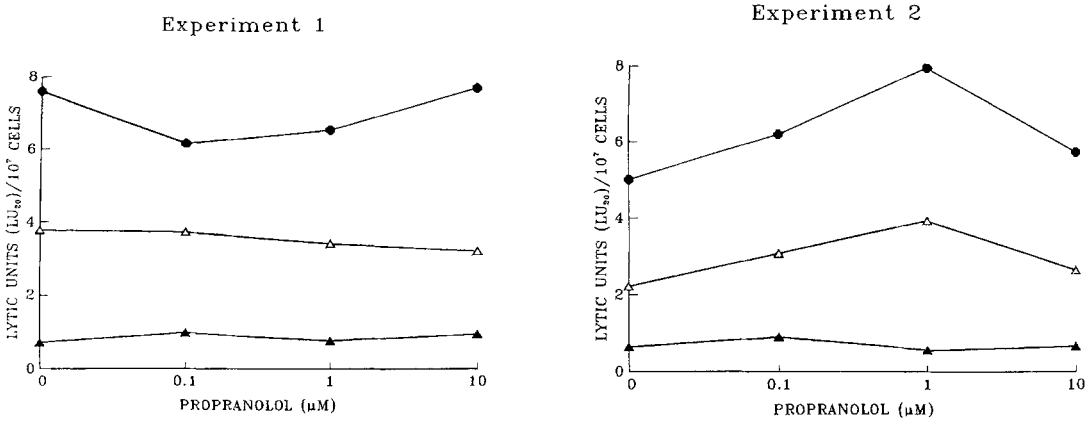


Fig. 6 Effect of propranolol on norepinephrine-mediated inhibition on the generation of anti-MOPC-315 cytotoxicity by normal BALB/c spleen cells. Spleen cells from BALB/c mice were incubated with propranolol for 30 min prior to the addition of mitomycin-C-treated MOPC-315 cells plus norepinephrine at either 50 μM (△) or 100 μM (▲). Controls (●) consisted of spleen cells incubated with mitomycin-C-treated MOPC-315 cells in the absence of norepinephrine. On day 5 after culture initiation, the spleen cells were evaluated for their lytic activity by the ^{51}Cr-release assay

The effect of catecholamines on the in vitro generation of CTL has been investigated in several other laboratories. These investigations focused on assessment of the effect of catecholamines on the generation of CTL in response to allogeneic stimulation and revealed the enhancing effects of catecholamines. Specifically, Hatfield et al. [15] have shown that norepinephrine (100 μM) and isoproterenol (0.1 μM) can enhance the in vitro generation of CTL activity in response to stimulation with allogeneic cells. Similarly, Felten et al. [12] have shown that adrenergic compounds in the nanomolar to micromolar concentration range enhanced the in vitro generation of an allogeneic CTL response. In the current study, we extend the previous observations by illustrating that the generation of a primary CTL response upon stimulation of normal BALB/c spleen cells with syngeneic MOPC-315 tumor cells can also be enhanced by the catecholamine norepinephrine at a low concentration (0.1–1.0 μM). However, no such norepinephrine-mediated enhancement in the in vitro generation of anti-MOPC-315 CTL response was observed with spleen cells from L-PAM TuB mice possibly because in vivo tumor progression followed by complete tumor regression (as a consequence of low dose L-PAM therapy) resulted in a maximal CTL response upon in vitro stimulation with MOPC-315 tumor cells thus obscuring any potential for norepinephrine-mediated enhancement.

An important contribution of the current investigation is the demonstration that, under defined conditions, catecholamine effects are concentration-dependent and can actually suppress the in vitro generation

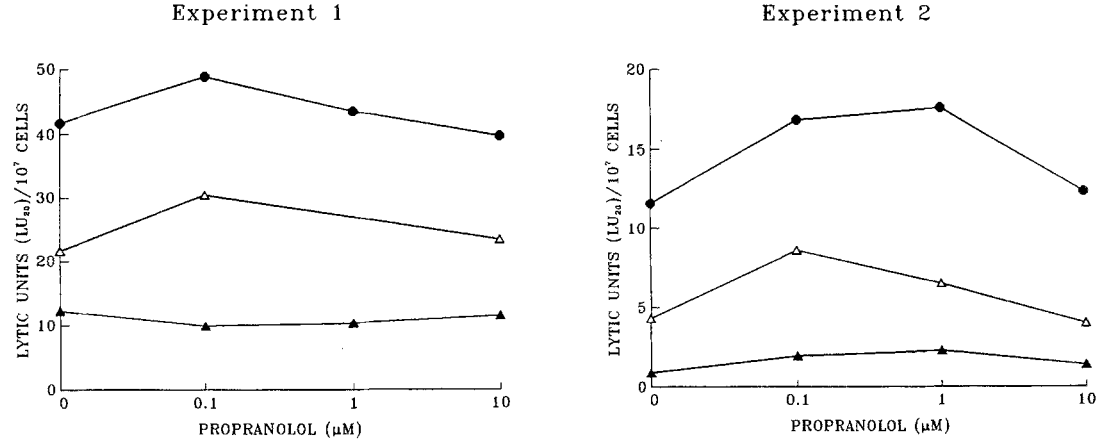


Fig. 7 Effect of propranolol on norepinephrine-mediated inhibition on the generation of anti-MOPC-315 cytotoxicity by spleen cells from L-PAM-treated MOPC-315 tumor beareries. Spleen cells from L-PAM BALB/c mice were incubated with propranolol for 30 min prior to the addition of mitomycin-C-treated MOPC-315 cells plus norepinephrine at either 50 μM (△) or 100 μM (▲). Controls (●) consisted of spleen cells incubated with mitomycin-C-treated MOPC-315 cells in the absence of norepinephrine. On day 5 after culture initiation, the spleen cells were evaluated for their lytic activity by the ^{51}Cr-release assay

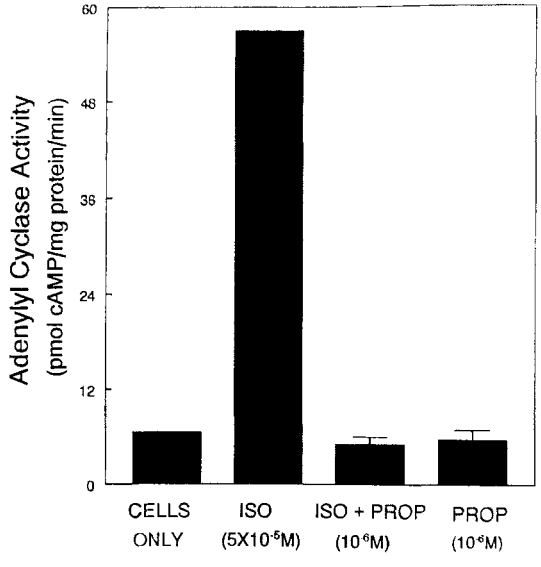


Fig. 8 Effect of propranolol on isoproterenol-stimulated adenylyl cyclase activity. Saponin-permeabilized BALB/c spleen cells were preincubated for 5 min with 1 μM propranolol (*PROP*) in the assay cocktail. Adenylyl cyclase was activated by the addition of 50 μM isoproterenol (*ISO*). The specific activity in cells is given for total cellular protein

of antitumor cytotoxicity. Specifically, in contrast to the observations of Hatfield et al. [15], where 100 μM norepinephrine enhanced the generation of a CTL response against allogeneic cells, our studies illustrate that at a concentration of approximately 100 μM the catecholamines norepinephrine, isoproterenol, and epinephrine drastically suppressed the in vitro generation of a CTL response against the syngeneic MOPC-315 plasmacytoma by normal spleen cells as well as by spleen cells from L-PAM TuB mice. The reason for this discrepancy between our findings and those of Hatfield et al. is as yet unknown. However, the suppression caused by the catecholamines in our studies is not due to nonselective toxicity of the drugs since (a) the catecholamines were inhibitory at concentrations that did not affect cell total viability or total cell yield (although it is still possible that the viability of a minor cell subpopulation may have been selectively sensitive to catecholamine-induced cytotoxicity) and (b) when the addition of the norepinephrine was delayed until day 3 after culture initiation, it did not lead to the generation of lower levels of anti-MOPC-315 cytotoxicity (data not shown). In fact, the concentration of norepinephrine that was inhibitory for the generation of anti-MOPC-315 cytotoxicity has been shown to be inhibitory for the proliferation of T cells in response to mitogenic stimulation [2, 5, 8, 12, 14, 20]. Thus, the possibility should be considered that this difference between our results and the results of Hatfield et al. [15] and Felten et al. [12] is a manifestation of differences in the sensitivity to catecholamine regulation of the generation of allogeneic compared to syngeneic CTL responses, similar to the differences in their requirement for interleukin-4 [36], and may reflect CTL differences in T-cell-receptor mediated transmembrane signalling which may result in the phosphorylation of different substrates through protein kinase A [32].

A major concern in the interpretation of the suppressive effects of catecholamines in these studies is whether such high concentrations of these agonists are associated with physiologically significant effects. The concentrations of catecholamines (approximately 100 μM) that were inhibitory for the generation of anti-MOPC-315 cytotoxicity are not unexpected for localized concentrations of norepinephrine in the spleens of stressed mice. The local concentration of

norepinephrine in the 6-nm synapse-like junctions between neurons and T lymphocytes is probably much higher than the 1 μM interstitial norepinephrine concentration in the normal mouse spleen [12]. The norepinephrine concentration in these synapse-like junctions with T cells also increases via sympathetic stimulation of splenic neurons during stress [29]. In addition, although the participation of the β-adrenergic receptors in the norepinephrine-mediated inhibition of the generation of anti-MOPC-315 cytotoxicity has not been demonstrated, the K_d (100–400 μM) for norepinephrine dissociation from β-adrenergic receptors present on lymphocytes [18, 37] is consistent with the catecholamine concentrations reported here to suppress the generation of antitumor cytotoxicity.

The catecholamine inhibition of the generation of anti-MOPC-315 cytotoxic activity was mimicked by cholera toxin, a stimulator of endogenous cAMP production, and by dibutyryl-cAMP, a membrane-penetrating analog of cAMP. Dibutyryl-cAMP inhibited the generation of other lymphocyte functions [6]. These observations, taken together with reports of isoproterenol stimulation of cAMP production in lymphocytes [9, 17, 23], are consistent with a cAMP-mediated mechanism for the catecholamine neurotransmitter-induced inhibition of the generation of anti-MOPC-315 cytotoxicity.

Here we show that norepinephrine exerted a bimodal effect on the generation of anti-MOPC-315 cytotoxicity by normal spleen cells, since the lower concentrations of norepinephrine augmented the response and the higher concentrations of norepinephrine reduced the response. The mechanism(s) through which norepinephrine exerts a bimodal effect on the generation of anti-MOPC-315 cytotoxicity is unknown. The possibility exists that the high concentrations of norepinephrine stimulate the production of high levels of cAMP, which inhibit the immune response, whereas low concentrations of norepinephrine stimulate the production of low levels of cAMP, which enhance the immune response. Consistent with this possibility are reports that cAMP effects on lymphocytes are concentration-dependent such that high concentrations of dibutyryl-cAMP inhibit lymphocyte proliferation [6, 34], whereas low concentrations of dibutyryl-cAMP appear to stimulate lymphocyte proliferation [34].

Propranolol, a β-adrenergic antagonist, did not prevent norepinephrine-induced inhibition of the generation of antitumor cytotoxicity, suggesting that classical β-adrenergic receptors may not be involved. Although cytotoxic T lymphocytes have β_2-adrenergic receptors [18, 27, 37] and isoproterenol binds to β_2-adrenergic receptors and stimulates adenylyl-cyclase-catalyzed cAMP production in lymphocytes [23], the relative potency of the agonists is not consistent with a typical β-adrenergic response in which isoproterenol would be expected to be at least two orders of magnitude more potent that norepinephrine. To test the role of β-receptors further, we used the antagonist propranolol. The inability of propranolol to block the effects of norepinephrine provides additional evidence for a non-classical mechanism of catecholamine action. This observation paralleled other results in our laboratory wherein propranolol failed to block norepinephrine-mediated inhibition of DNA synthesis by splenic B and T cells, as well as by the S49 T-cell lymphoma cell line [5]. However, even though propranolol did not alter the norepinephrine-induced inhibition of the generation of antitumor cytotoxicity, it is possible that the norepinephrine-inhibitory effects are mediated through a cAMP-dependent mechanism. Such an argument is consistent with our previous studies with the S49 mutants wherein dibutyryl-cAMP, but not norepinephrine, inhibited proliferation of S49 mutants lacking adenylyl cyclase, while neither dibutyryl-cAMP nor norepinephrine could inhibit proliferation of an S49 mutant lacking functional protein kinase A activity. These data suggest that norepinephrine signal transduction is primarily through an adenylyl-cyclase/cAMP/protein-kinase-A mechanism [5]. The complexities of protein-kinase-A-mediated phosphorylation of substrates at the levels of cell surface receptors, transcription factors and translation of regulatory lymphokines (e.g., interleukin-2) and cytoplasmic proteins related to cytotoxicity (e.g., perforins) have yet to be understood for CTL.

The ability of the catecholamine norepinephrine to inhibit the in vitro generation of antitumor cytotoxicity by L-PAM TuB spleen cells suggests that stress-induced elevation of norepinephrine levels could down-regulate the in vivo acquisition of CTL activity by T cells from mice bearing a large MOPC-315 tumor and treated with low-dose L-PAM. At this time, it is not known if norepinephrine-mediated inhibition of the generation of antitumor cytotoxicity by L-PAM TuB spleen cells is a function of direct or indirect effects of catecholamines on $CD8^+$ T cells. Thus, norepinephrine-mediated inhibition of the generation of antitumor cytotoxicity may, in turn, affect the therapeutic outcome of low-dose L-PAM therapy, since the low dose of the drug depends on the $CD8^+$ T-cell-dependent antitumor immunity for the eradication of a large tumor burden [22, 33], and reduction in the acquisition of anti-MOPC-315 CTL activity by the $CD8^+$ T cells will be manifested by reduction in the effectiveness of tumor-eradicating immunity [22, 33].

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