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Neurotransmitter Suppression of  
the In Vitro Generation of the Cytotoxic T-lymphocyte Response  
Against Syngeneic MOPC-315 Plasmacytomas by  
Spleen Cells from Melphalan-cured MOPC-315 Tumor Bearers<sup>1</sup>

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Running Title: Catecholamine Inhibition of Tumor Immunity  
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## Abstract

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The effects of the "stress"-related mediator norepinephrine and related compounds on the generation of anti-tumor cytotoxic activity by spleen cells from melphalin-cured MOPC-315 tumor bearing mice were determined. Cytotoxic activity was generated by coculture of spleen lymphocytes from melphalin-cured MOPC-315 tumor bearing BALB/c mice with syngeneic MOPC-315 plasmacytoma cells and was assayed by the  $^{51}\text{Cr}$  release assay. At concentrations of 50-100  $\mu\text{M}$ , the catecholamines, norepinephrine, epinephrine, and isoproterenol, and the non-catecholamine, dopamine inhibited the development of cytotoxic activity by 30-96%. Generation of anti-MOPC-315 cytotoxicity was not significantly affected by serotonin or carbachol. The generation of cytotoxic activity during a 5 to 9 day coculture period was inhibited by norepinephrine (50-100  $\mu\text{M}$ ) when it was added up to 2 days after the initiation of the culture while dibutyryl cAMP suppressed a 5 day generation of cytotoxic activity when added 0-3 days after culture initiation. In contrast to the effects by norepinephrine, the level of inhibition of the generation of cytotoxic activity by dibutyryl cAMP, when added on day 4, was greater 9 days after culture initiation as compared to the inhibition of cytotoxic activity on day 5 after culture initiation. The  $\beta$ -adrenergic antagonist propranolol did not alter the inhibitory effects of catecholamines on the generation of cytotoxicity. Catecholamine-induced suppression of the generation of anti-tumor cytotoxic activity by lymphocytes from melphalin-

cured MOPC-315 tumor bearers may help to explain the effects of stress on tumor progression, eradication, and the clinical outcome of cancer patients following chemotherapy.

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## Introduction

Tumor progression, tumor eradication, and the clinical outcome of cancer treatment varies greatly among individuals. These variations appear to relate, in part, to the detrimental effects of psychological and physiological stress on immune function. Fawzy et al. (1, 2) reported that psychiatric intervention reduced psychologic distress, enhanced longer-term coping and enhanced the numbers and cytotoxic activity of natural killer (NK) cells in patients with malignant melanoma. Other investigations associate surgical, environmental, physiological, and psychosocial stress with impaired antitumor immunity (3, 4), suggesting that such stress may reduce the generation of antitumor immunity through a mechanism(s) involving activation of the sympathetic nervous system and the release of norepinephrine and/or other sympathetic neurotransmitters (5).

Lymphoid organs are innervated by the sympathetic nervous system and synapse-like junctions occur between lymphocytes and neurons (6) indicating that lymphocytes are exposed to neurotransmitters in their reservoir organs. Norepinephrine is of particular interest since it is the major sympathetic neurotransmitter, it is present in high concentration in the spleen (6), and it plays a central role in the response of the sympathetic nervous system to stress. Immunoregulation by norepinephrine in vivo is suggested by the observation that sympathetic denervation of the spleen enhances antibody production (7-9), albeit through

undefined mechanisms. Consistent with these in vivo studies catecholamine neurotransmitters have also been shown to inhibit the proliferation of B-lymphocytes and T-lymphocytes in vitro (10-12), NK activity (13), and cytolysis of tumor targets by cytotoxic T-lymphocytes (14). In our previous studies, it was observed that there are different mechanisms of catecholamine inhibition in B cells and T cells (10). The catecholamine inhibition of T and B-cell DNA synthesis was not blocked by adrenergic or dopaminergic receptor antagonists (J.M. Cook-Mills, M. Mokyr, R.L. Perlman, and D.A. Chambers, submitted). Catecholamines also inhibit the in vitro generation of MOPC-315 tumor cell-stimulated anti-MOPC-315 cytotoxicity by spleen cells from normal mice (J.M. Cook-Mills, et. al., submitted).

In light of the evidence for stress-induced immunosuppression in cancer patients (1-5), immune responses to tumor cells by individuals who have undergone chemotherapy may also be regulated by stress-related molecules. The current study was undertaken to determine whether stress-related neurotransmitters can inhibit the generation of a potent cytotoxic T-lymphocyte (CTL) response against a syngeneic tumor by spleen cells from melphalan-cured tumor bearing mice. The focus of our attention on the generation of CTL activity against tumor cells arises from the growing appreciation of the importance of the CTL lytic mechanism in tumor eradication (15-20). As a prototype of a tumor model in which the CTL plays an important role in in vivo tumor eradication, we selected the murine MOPC-315 tumor model (21, 22). In previous

studies employing MOPC-315 tumors, chemotherapy with a low dose of the drug melphalin enhanced the appearance of potent antitumor immunity and eradication of large (20-22 mm) MOPC-315 subcutaneous tumors (23). The MOPC-315 tumor model also provides an ideal system to study the effects of catecholamines on the generation of antitumor cytotoxicity in vitro since the exact conditions for the in vitro generation of a CTL response against MOPC-315 tumor cells by spleen cells from melphalin-cured tumor bearing mice are well established (23). In this study, the MOPC-315 tumor system was used to examine the effects of catecholamines, non-catecholamine neurotransmitters and associated molecules on the generation of a CTL response in vitro by spleen cells from melphalin-cured MOPC-315 tumor bearing mice.

## Materials and Methods

Animals. Animals used for maintenance of tumor cells and as a source of spleen cells for the in vitro generation of cytolytic activity were 7 to 10 week old female BALB/c mice purchased from Charles River Breeding Laboratories, Wilmington, MA.

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

Tumors. MOPC-315 cells were maintained in vivo as a subcutaneous tumor as previously described (24). Routinely, mice were inoculated subcutaneously with  $1 \times 10^6$  viable tumor cells which 10 days later resulted in a tumor of 20-22 mm in diameter. Single cell suspensions were prepared by mechanical disruption of the tumor between glass slides (24). The viability, as determined by trypan blue dye exclusion (0.4%), always exceeded 95%.

Chemotherapy. A fresh stock solution of 10 mg melphalan (Burroughs Wellcome Co., Triangle Park, NC)/ml was prepared just prior to injection as previously described (22) and was further diluted with Dulbecco's phosphate buffered saline, pH 7.2 (Grand Island Biological Co., Grand Island, NY), to the desired concentration. A dose of 2.5 mg of melphalan per kg of body weight (low dose) was immediately administered intraperitoneally to mice bearing a subcutaneous MOPC-315 tumor with a diameter of 20-22 mm resulting from the inoculation of  $1 \times 10^6$  MOPC-315 tumor cells 10 days earlier. This dose of drug was chosen because it is the lowest dose of melphalan which is curative for at least 90% of mice

bearing a large ( $\geq 20$  mm) MOPC-315 tumor (22).

Spleen Cell Suspensions. Single cell suspensions from spleens from melphalan-cured BALB/c mice were prepared by mincing and gently pressing the cells through a sterile Nytex nylon mesh (Tetko, Inc., Emsford, NY) with a sterile stainless steel lab spoon (American Scientific Products, McGaw Park, IL). The single cell suspension was washed and resuspended in culture medium for use in the in vitro stimulation. In any single experiment, pooled spleen cells from at least 5 melphalin-cured mice were used.

In Vitro Stimulation for the Generation of Cytotoxic T-lymphocytes. MOPC-315 tumor cells from MOPC-315 tumor bearers were excised and processed to a single cell suspension by mechanical disruption, as previously described (24). The viability of the cells, as determined by trypan blue dye exclusion (0.4%), always exceeded 95%. The MOPC-315 tumor cells were treated with mitomycin C (50  $\mu\text{g/ml}$ ) for 30 minutes and washed three times. Mitomycin C-treated tumor cells ( $1.33 \times 10^6$ ) were admixed with spleen cells ( $40 \times 10^6$ ) from melphalan-cured MOPC-315 tumor bearing BALB/c mice and cultured in 20 ml of medium consisting of RPMI 1640 supplemented with 1% nonessential amino acids (#320-1140, Gibco, Grand Island, NY), 2 mM glutamine, 50  $\mu\text{M}$  2-mercaptoethanol (Sigma, St. Louis, MO), 50 Units penicillin, 50  $\mu\text{g/ml}$  streptomycin (Sigma) and 5% heat-inactivated fetal bovine serum (Gibco). Agonists were added at the time of culture initiation unless otherwise stated. The cultures were incubated at 37°C in 5%CO<sub>2</sub>-air for 5 days unless otherwise stated since the generation of the CTL response peaks

between days 4-6 (24, 25).

Cytolytic Assay. The cytolytic activity of the cultured spleen cells was determined by the  $^{51}\text{Cr}$  release assay as previously described (24). Briefly, the cultured spleen cells were washed three times and incubated for 3.5 hours with  $5 \times 10^4$   $^{51}\text{Cr}$ -labelled target MOPC-315 cells in 12 x 75 mm plastic tubes at effector to target cell (E/T) ratios of 100:1, 50:1, and 25:1. At the end of the incubation period, the cells were pelleted, and radioactivity in the supernatants (Sup) and pellets (Pel) was counted in a gamma scintillation counter. The percentage of specific  $^{51}\text{Cr}$  release for each sample was calculated as follows:

$$\% \text{ of } ^{51}\text{Cr} \text{ release} = \frac{\text{cpm in Sup.}}{\text{cpm in Sup.} + \text{cpm in Pel.}} \times 100$$

The percentage of specific  $^{51}\text{Cr}$  release was calculated by the following formula:

$$\% \text{ of specific } ^{51}\text{Cr} \text{ release} = \frac{T - C}{M - C} \times 100$$

where T is the percentage of release with test lymphocytes, C is the mean value calculated for three replicates of the percentage of spontaneous release (which ranged between 16 and 30%), and M is the mean value calculated for three replicates of the percentage of maximal release (obtained after addition of 2% NP-40), which ranged between 82 and 97%. Each experiment was performed a minimum of two times. The level of antitumor cytotoxicity is presented as the mean  $\pm$  SEM from triplicate samples. The SEM did not exceed 6%. Although the level of antitumor cytotoxicity generated by lymphoid

cells varied from one experiment to another (26-28), the pattern of the results remained consistent and reproducible. Probability values of  $p < 0.05$  as determined by a completely random Anova followed by Dunnett's t Test were considered statistically significant.

## Results

Catecholamine Agonist Inhibition of the Generation of Anti-MOPC-315 Cytotoxic Activity by Spleen Cells from Melphalin-cured MOPC-315 Tumor Bearing Mice. The effects of catecholamines on the generation of a cytotoxic T-cell response against a syngeneic tumor were examined. Norepinephrine, epinephrine, or isoproterenol was added to spleen cells from melphalin-cured MOPC-315 tumor bearing female BALB/c mice at the time of initiation of a 5 day culture with mitomycin-C-treated MOPC-315 tumor cells. As a control, spleen cells were cultured with MOPC-315 cells in the presence of HCl, the solvent for the neurotransmitters. As a control, spleen cells were cultured with MOPC-315 cells in the presence of HCl. At the concentrations used, HCl had no effect on the generation of cytotoxic activity (Fig.1). At high concentrations (100  $\mu$ M), all of the catecholamines inhibited (30-96% inhibition) the generation of anti-MOPC-315 cytotoxicity (Fig.1). The extent of inhibition by norepinephrine, epinephrine, and isoproterenol varied between experiments; however, it was consistent that these catecholamines (50-100  $\mu$ M) significantly inhibited the generation of anti-MOPC-315 cytotoxicity. Figure 1 shows that the inhibition was evident when the lytic activity was assayed at effector to target cell (E/T) ratios of 100:1, 50:1 and 25:1. The cytotoxicity exerted by the norepinephrine-treated cells at an E/T ratio of 100:1 was lower than that exerted by control cells at an E/T ratio of 25:1. The dramatically reduced anti-tumor cytotoxicity following stimulation

in the presence of the catecholamines was not a consequence of drug toxicity since the drugs did not affect cell viability as determined by trypan blue exclusion and by fluorescein diacetate fluorescence (measured after 2 days of culture) nor did they reduce the total number of lymphocytes harvested on day 5. Thus, stress-related molecules decreased the generation of anti-tumor activity by spleen cells from melphalin-cured MOPC-315 tumor bearing mice. Concentrations of norepinephrine below 1  $\mu\text{M}$  did not affect the generation of anti-MOPC-315 cytotoxicity (data not shown).

The Effects of Dopamine on the Generation of Anti-tumor Cytotoxic Activity. Since dopamine can act through its own dopamine receptors (29) as well as adrenergic receptors, the effects of dopamine on the 5 day generation of antitumor cytotoxicity was also investigated. Figure 2 reveals that dopamine (75-100  $\mu\text{M}$ ) inhibited the generation of anti-MOPC-315 cytotoxicity was drastically by 68-82%. The decrease in generation of antitumor cytotoxicity with dopamine (50-100  $\mu\text{M}$ ) was not due to toxicity since dopamine did not affect the cell yield on day 5 of the culture.

The Effects of Non-catecholamine Neurotransmitters on the Generation of Anti-tumor Cytotoxic Activity. Experiments were performed to determine whether non-catecholamine neurotransmitters could effect the generation of anti-MOPC-315 cytotoxicity. When serotonin (a non-catecholamine), or carbachol (an acetylcholine agonist) were added at the initiation of a 5 day in vitro generation of anti-tumor activity, inhibition of the generation of

cytotoxic activity against MOPC-315 tumor cells was observed (Fig.2).

Cyclic-AMP Parallels the Catecholamine Inhibition of the Generation of Anti-tumor Cytotoxicity. Our previous studies suggest that cAMP is the second messenger for catecholamines in T cells (10). To investigate the role of cAMP as a second messenger in the inhibition of lymphocyte activation by catecholamines, we examined whether dibutyryl cAMP, a membrane penetrable analog of cAMP, could act in ways similar to catecholamines. Figure 3 reveals that, at concentrations previously shown to inhibit other lymphocyte functions (30), dibutyryl cAMP did indeed inhibit the generation of anti-MOPC-315 cytotoxic activity by spleen cells from melphalin-cured tumor bearing mice. Thus, cAMP, like the catecholamines, can inhibit the generation of anti-MOPC-315 cytotoxicity. Since inhibition by norepinephrine may be mediated via cAMP it was important to determine if both compounds have the same kinetics of inhibition. Norepinephrine or dibutyryl cAMP was added to spleen cells immediately prior to addition of the mitomycin-C-treated MOPC-315 tumor cells. Cytotoxic activity against MOPC-315 cells was assessed on days 3, 4, and 5 of the culture. Similar levels of cytotoxicity were obtained by a 3, 4, and 5 day culture (67-76% specific <sup>51</sup>Cr release, data not shown). Figure 4 shows that norepinephrine and dibutyryl cAMP inhibited the generation of cytotoxic activity to the same extent at all three time points.

Effect of Delayed Addition of Norepinephrine and Dibutyryl

cAMP on the Generation of Anti-tumor Cytotoxic Activity.

Experiments were performed to determine whether norepinephrine and cAMP inhibit the same "stage" of the generation of anti-tumor cytotoxicity. Accordingly, we determined whether norepinephrine and dibutyryl cAMP affects only early or later events in the generation of antitumor cytotoxicity. Norepinephrine or dibutyryl cAMP was added to spleen cells and mitomycin-C-treated MOPC-315 cells on days 0, 1, 2, 3, or 4 and cytotoxic activity was assessed simultaneously for all treatment groups on 5 day of the culture. Figure 5 illustrates that maximal inhibition of the generation of cytotoxic activity occurred upon addition of norepinephrine on day 0 of the *in vitro* stimulation, substantially less inhibition was noted when norepinephrine was added on day 1 or 2, and no inhibition could be seen when it was added after day 3. Generation of cytotoxic activity was inhibited by dibutyryl cAMP when it was added up to 3 days after culture initiation.

Since there may be a 5 day requirement for maximal exertion of an inhibitory effect by norepinephrine and dibutyryl cAMP, cytotoxic activity was determined 5 days after the delayed addition of norepinephrine and dibutyryl cAMP. Mitomycin-C-treated MOPC-315 tumor cells were added to spleen cells from melphalin-cured MOPC-315 tumor bearing mice. Norepinephrine and dibutyryl cAMP was added on day 0, 1, 2, 3, or 4 followed by analysis of cytotoxic activity on days 5, 6, 7, 8, and 9, respectively. Figure 6 shows that addition of norepinephrine on day 3 or 4 (arrows in Fig. 6) followed by analysis of cytotoxicity on days 8 and 9, respectively,

was not inhibitory. This lack of inhibition was similar to that when norepinephrine was added on days 3 or 4 and cytotoxicity analyzed on day 5 (Fig. 5). In contrast, figure 6 shows that addition of dibutyryl cAMP on day 4 was inhibitory when cytotoxic activity was measured on day 9 but was not inhibitory when cytotoxicity was measured on day 5 (Fig. 5) suggesting that 5 days are required for dibutyryl cAMP to manifest its inhibition of the generation of cytotoxic activity by spleen cells from melphalin-cured MOPC-315 tumor bearers.

Analysis of the Participation of the  $\beta$ -Adrenergic Receptor in Catecholamine Inhibition of the Generation of Cytotoxic Activity.

Since adenylyl cyclase which catalyzes the production of cAMP is coupled to  $\beta$ -adrenergic receptors, experiments were designed to determine whether catecholamines inhibited the generation of cytotoxic activity via binding to  $\beta$ -adrenergic receptors found on lymphocytes (31-33). For this purpose, the ability of propranolol, a  $\beta$ -adrenergic antagonist, to block the inhibitory effects of norepinephrine on the generation of antitumor cytotoxicity was studied. Spleen cells from melphalin-cured MOPC-315 tumor bearing mice were incubated with propranolol for 30 minutes prior to addition of norepinephrine and mitomycin-C-treated MOPC-315 tumor cells. After 5 days in culture, cytotoxic activity was measured. Figure 7 shows that propranolol (0.1-10  $\mu$ M) did not block the norepinephrine-induced (100  $\mu$ M) inhibition of the generation of anti-MOPC-315 cytotoxicity by spleen cells from melphalin-cured MOPC-315 tumor bearing mice. This lack of antagonistic effect by

propranolol was not the result of a non-functional propranolol compound since propranolol blocked isoproterenol stimulation of adenylyl cyclase activity by the mixed population of cells from normal mouse spleens (data not shown), a result observed previously (Cook-Mills, J.C., Hayden, R., Perlman, R.L., Chambers, D.A., submitted 1991).

## Discussion

It has been demonstrated that the anti-MOPC cytotoxicity is due to MOPC-315 specific CD8<sup>+</sup> CTL cells generated in vitro (34-37). The studies in this report demonstrate that the catecholamines isoproterenol, norepinephrine, and epinephrine but not the non-catecholamines serotonin and carbachol can suppress the in vitro generation of a CTL response against the syngeneic MOPC-315 plasmacytoma by spleen cells from melphalin-cured MOPC-315 tumor bearing mice. These molecules were inhibitory at concentrations of 75-100  $\mu\text{M}$ , nontoxic concentrations not unexpected for localized concentrations of norepinephrine in the spleens of stressed mice. The concentration of norepinephrine in the 6 nm synapse-like junctions between neurons and T-lymphocytes is probably much higher than the 1  $\mu\text{M}$  interstitial norepinephrine concentration in mouse spleen (6). Moreover, under stress, sympathetic stimulation increases norepinephrine release in the spleen (38). Furthermore, although the participation of the known  $\beta$ -adrenergic receptors in the inhibition of anti-MOPC-315 cytotoxicity is unclear, the  $K_D$  for norepinephrine dissociation from  $\beta$ -adrenergic receptors present on lymphocytes (100-400  $\mu\text{M}$ ) is similar to the "effector" catecholamine concentrations reported here (31, 32). The catecholamine-specific inhibition of the generation of anti-MOPC-315 cytotoxicity was also observed when using this tumor cell system with spleen cells from normal mice (J.M. Cook-Mills, M. Mokyr, R.L. Perlman, and D.A. Chambers, submitted). Thus, not only do catecholamines inhibit the

generation of anti-tumor immunity by spleen cells from normal mice but they also inhibited the generation of the potent anti-tumor response of spleen cells from mice that had undergone chemotherapy.

The catecholamine inhibition of the generation of anti-MOPC-315 cytotoxic activity by spleen cells from melphalin-cured MOPC-315 tumor bearers was mimicked by dibutyryl cAMP, a membrane penetrable analog of the adrenergic second messenger cAMP. Dibutyryl cAMP inhibited the generation of cytotoxicity at concentrations (10  $\mu$ M and 500  $\mu$ M) that inhibit other immune responses such as T-cell DNA synthesis (10, 12, 30, 39) and the generation of anti-MOPC-315 cytotoxicity by spleen cells from normal mice (manuscript submitted for publication). Such concentrations of dibutyryl cAMP appear to be physiologically relevant since lymphocytes stimulated with isoproterenol or cholera toxin contain endogeneous cAMP concentrations of 11-40 pmoles cAMP/ $10^6$  cells which we calculated as 3.5-13  $\mu$ M cAMP assuming an average lymphocyte diameter of 9  $\mu$ m which translates into a cell volume of  $3 \times 10^{-9}$  ml (33, 40-43). Our results, taken together with reports of isoproterenol stimulation of cAMP production in lymphocytes (33, 40-42), are consistent with a cAMP-mediated mechanism for catecholamine neurotransmitter-mediated inhibition of the generation of anti-MOPC-315 cytotoxicity by spleen cells from melphalin-cured MOPC-315 tumor bearing mice.

However, norepinephrine and dibutyryl cAMP inhibition was evident when added at early events (day 0-2) in the 5 day generation of cytotoxicity. The lack of norepinephrine inhibition

when added on day 3 or 4 with analysis of cytotoxicity on day 5 was not overcome by a longer culture incubation and analysis of cytotoxicity on day 8 and 9, whereas the lack of inhibition by day 4 treatment with dibutyryl cAMP was overcome when cytotoxicity was analyzed on day 9 instead of day 5. A number of possible explanations which could account for the inability of norepinephrine to down regulate the later events in the generation of cytotoxicity include reduced receptor numbers and/or affinity, uncoupling of receptors to adenylate cyclase, or use of a second cAMP signal transduction pathway(s) independent of norepinephrine.

Propranolol, a classical  $\beta$ -adrenergic antagonist, did not prevent norepinephrine-induced inhibition of the generation of cytotoxicity suggesting that  $\beta$ -adrenergic receptors may not be involved. The inability of propranolol to block the effects of norepinephrine was unexpected since cytotoxic T-lymphocytes have  $\beta_2$ -adrenergic receptors (31, 32), isoproterenol binds to  $\beta_2$ -adrenergic receptors and stimulates cAMP production in lymphocytes (40), cAMP inhibits the generation of anti-tumor immunity by spleen cells from normal mice (J.M. Cook-Mills, et. al., submitted), and, as described in this report, cAMP inhibited the generation of anti-tumor immunity by spleen cells from melphalin-cured tumor bearing mice. However, the possibilities exist that under our experimental conditions, the  $\beta$ -adrenergic receptor may escape the antagonistic effects of propranolol or that catecholamines may act via a non-receptor-mediated mechanism, a propranolol insensitive  $\beta$ -adrenergic receptor, or an as yet undefined receptor, an alternative

consistent with recent reports of the presence of novel neurotransmitter receptors on lymphocytes which appear to act in non-defined ways (33, 44).

The inability of propranolol to block the inhibition of the generation of anti-MOPC-315 cytotoxicity by catecholamines is similar to our previous results with parallel studies on spleen B-cell and T-cell replication, thymic cell replication and S49 T-lymphoma replication (submitted 1991). Our previous studies with the S49 mutants showed that dibutyryl cAMP but not norepinephrine inhibited proliferation by S49 mutants lacking adenylate cyclase and that neither dibutyryl cAMP or norepinephrine inhibited proliferation by the S49 mutant lacking protein kinase A activity suggesting that norepinephrine signal transduction was primarily through an adenylate cyclase-cAMP-protein kinase A mechanism (submitted 1991). Since receptors other than  $\beta$ -adrenergic receptors activate adenylate cyclase for the production of cAMP (45-48), perhaps the norepinephrine-mediated inhibition of the generation of cytotoxicity is through a receptor which stimulates cAMP production other than the  $\beta$ -adrenergic receptor. There is also the possibility that the catecholamines act via an  $\alpha$ -adrenergic receptor. However, in our previous studies,  $\alpha$ -adrenergic antagonists or dopaminergic antagonists did not block norepinephrine inhibition of mitogen-stimulated lymphocyte DNA synthesis (J.M. Cook-Mills, et. al., submitted).

In this report, we have demonstrated that catecholamines inhibit the generation of anti-MOPC-315 cytotoxicity. The effect

of catecholamines is not limited to inhibition of the generation of antitumor cytotoxicity. In fact, catecholamines can inhibit expression of the lytic activity by fully activated CTL's. Henney et al. (14) showed that the secretory phase of target cell lysis is inhibited by isoproterenol (0.1-100  $\mu\text{M}$ ) and cAMP (500  $\mu\text{M}$ ). Therefore, catecholamines inhibit both initial events in the generation of cytotoxic activity, as described herein, and degranulation of CTL's (14). The catecholamine inhibition of the generation and expression of CTL activity in vitro suggests that stress-induced elevation of catecholamine levels may inhibit tumor eradication in vivo by down regulating the generation of CTL activity as well as by down regulating the delivery of the lethal hit by the CTL's. In addition, the catecholamine inhibition of the generation of CTL activity in vitro by spleen cells from melphalin-cured MOPC-315 tumor bearers suggests that stress-induced production of catecholamines in vivo may reduce an immune response by chemotherapy-cured individuals to a second challenge by a tumor. Our studies describing the inhibition of antitumor cytotoxicity supply the framework for further studies of the mechanisms for stress-induced modulation of the generation of antitumor immunity.

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## Figure Legends

Figure 1. Concentration Curves for Catecholamine Modulation of the In Vitro Generation of Anti-MOPC-315 Cytotoxicity by BALB/c Spleen Cells. Norepinephrine (*closed triangle, dashed line*), epinephrine (*closed square, dotted line*), isoproterenol (*closed diamond, dashed/dotted line*), or the HCl solvent control (*closed circle, solid line*) was added to spleen cells from melphalin-cured MOPC-315 tumor bearing BALB/c mice admixed with mitomycin-C-treated syngeneic MOPC-315 plasmacytoma cells for 5 days. The in vitro stimulated cells were evaluated for their lytic activity at effector/target cell (E/T) ratios of (A) 100:1, (B) 50:1, and (C) 25:1. Data are from a representative experiment of three experiments. Where error bars are not shown, the error bars are smaller than the symbol. \*, Statistical significance ( $p < 0.05$ ) relative to lytic activity exhibited by spleen cells stimulated with MOPC-315 cells in the absence of agonists.

Figure 2. Concentration Curves for Modulation of the Generation of Anti-MOPC-315 Cytotoxicity by Dopamine, Carbachol, and Serotonin. Dopamine (*closed triangle, dashed line*), serotonin (*closed square, dotted line*), carbachol (*closed diamond, dashed/dotted line*) or HCl (*closed circle, solid line*) was added with spleen cells from melphalin-cured MOPC-315 tumor bearing BALB/c mice admixed with

mitomycin-C-treated syngeneic MOPC-315 plasmacytoma cells for 5 days. The in vitro immunized cells were evaluated for their lytic activity at E/T ratios of (A) 100:1, (B) 50:1, and (C) 25:1. Data are from the same representative experiment shown in figure 1. Where error bars are not shown, the error bars are smaller than the symbol. \*, Statistical significance ( $p < 0.05$ ) relative to lytic activity of spleen cells incubated with mitomycin-C-treated MOPC-315 cells.

Figure 3. Concentration Curve for Dibutyryl cAMP Inhibition of the Generation of Anti-MOPC-315 Cytotoxicity by Spleen Cells from Melphalin-cured MOPC-315 Tumor Bearers. Dibutyryl cAMP was incubated with spleen cells from melphalin-cured MOPC-315 tumor bearing BALB/c mice admixed with mitomycin-C-treated syngeneic MOPC-315 plasmacytoma cells for 5 days followed by assessment of lytic activity. The data shown are the lytic activity of in vitro immunized cells at an effector/target cell ratio of 100:1. Data are from a representative experiment of more than three experiments. \*, Statistical significance ( $p < 0.05$ ) relative to lytic activity of spleen cells incubated with mitomycin-C-treated MOPC-315 cells.

Figure 4. Extent of Inhibition of Anti-MOPC-315 Cytotoxicity by Norepinephrine and Dibutyryl cAMP after 3, 4, or 5 days. Norepinephrine (NE), dibutyryl cAMP (DBcAMP), or the HCl

solvent control was incubated with spleen cells from melphalin-cured MOPC-315 tumor bearing BALB/c mice admixed with mitomycin-C-treated MOPC-315 cells for 3, 4, or 5 days followed by assessment of lytic activity. The data shown are the percent inhibition of lytic activity of in vitro immunized cells at an effector/target cell ratio of 100:1. Data are from a representative experiment of two experiments. \*, Statistical significance ( $p < 0.05$ ) relative to the percent inhibition of lytic activity of spleen cells incubated mitomycin-C-treated MOPC-315 cells in the presence of HCl (100  $\mu$ M).

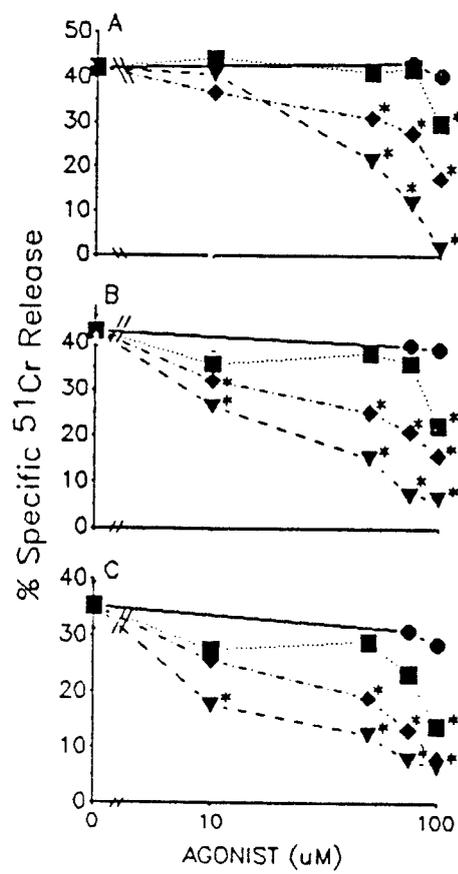
Figure 5. Effect of Temporal Addition of Norepinephrine and Dibutyryl cAMP on the Generation of Anti-MOPC-315 Cytotoxicity by Spleen Cells from Melphalin-cured MOPC-315 Tumor Bearers. Spleen cells from melphalin-cured MOPC-315 tumor bearing BALB/c mice were cocultured with mitomycin-C-treated MOPC-315 cells. On days 0-4 after culture initiation, norepinephrine (100  $\mu$ M, closed diamond, dashed/dotted line), dibutyryl cAMP (500  $\mu$ M, open diamond, dashed line), or the HCl solvent control (closed circle, solid line) were added to the cultures. On day 5, the in vitro immunized cells were evaluated for their lytic activity at effector/target cell ratio of 100:1. Data are from a representative experiment of three experiments. Where error bars are not shown, the error bars are smaller than the symbol. \*, Statistical

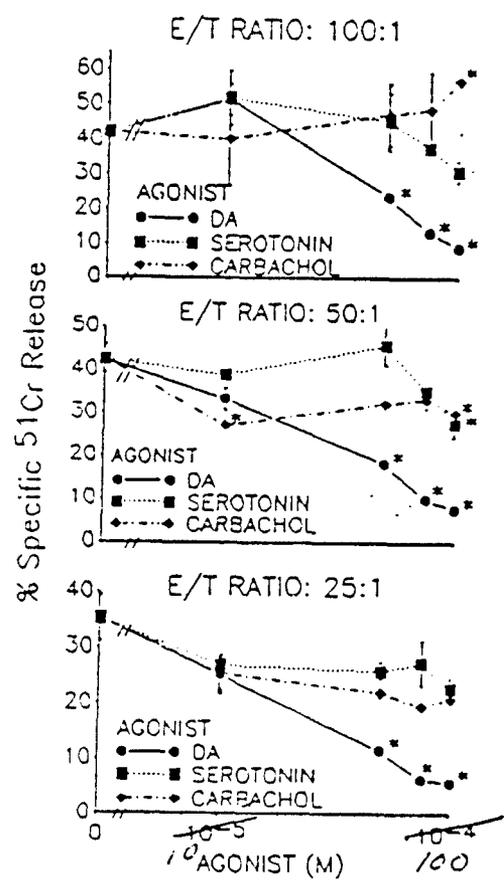
significance relative to lytic activity of spleen cells incubated with HCl (100  $\mu$ M) and mitomycin-C-treated MOPC-315 cells.

Figure 6. Effect of Norepinephrine and Dibutyryl cAMP on the Generation of Cytotoxic Activity 5 Days after Temporal Addition of Norepinephrine and Dibutyryl cAMP. Spleen cells from melphalin-cured MOPC-315 tumor bearing mice were cocultured with mitomycin-C-treated MOPC-315 tumor cells. Norepinephrine (NE), dibutyryl cAMP (DBcAMP), and the HCl solvent control were added to the cultures on day 0, 1, 2, 3, or 4 and, on day 5, 6, 7, 8, and 9, respectively, the in vitro immunized cells were evaluated for their lytic activity.  $\psi$ , indicates the time of norepinephrine and dibutyryl cAMP addition to the in vitro coculture. Data are from the same representative experiment shown in Fig. 5. \*, Statistical significance relative to lytic activity of spleen cells incubated with HCl (100  $\mu$ M) and mitomycin-C-treated MOPC-315 tumor cells.

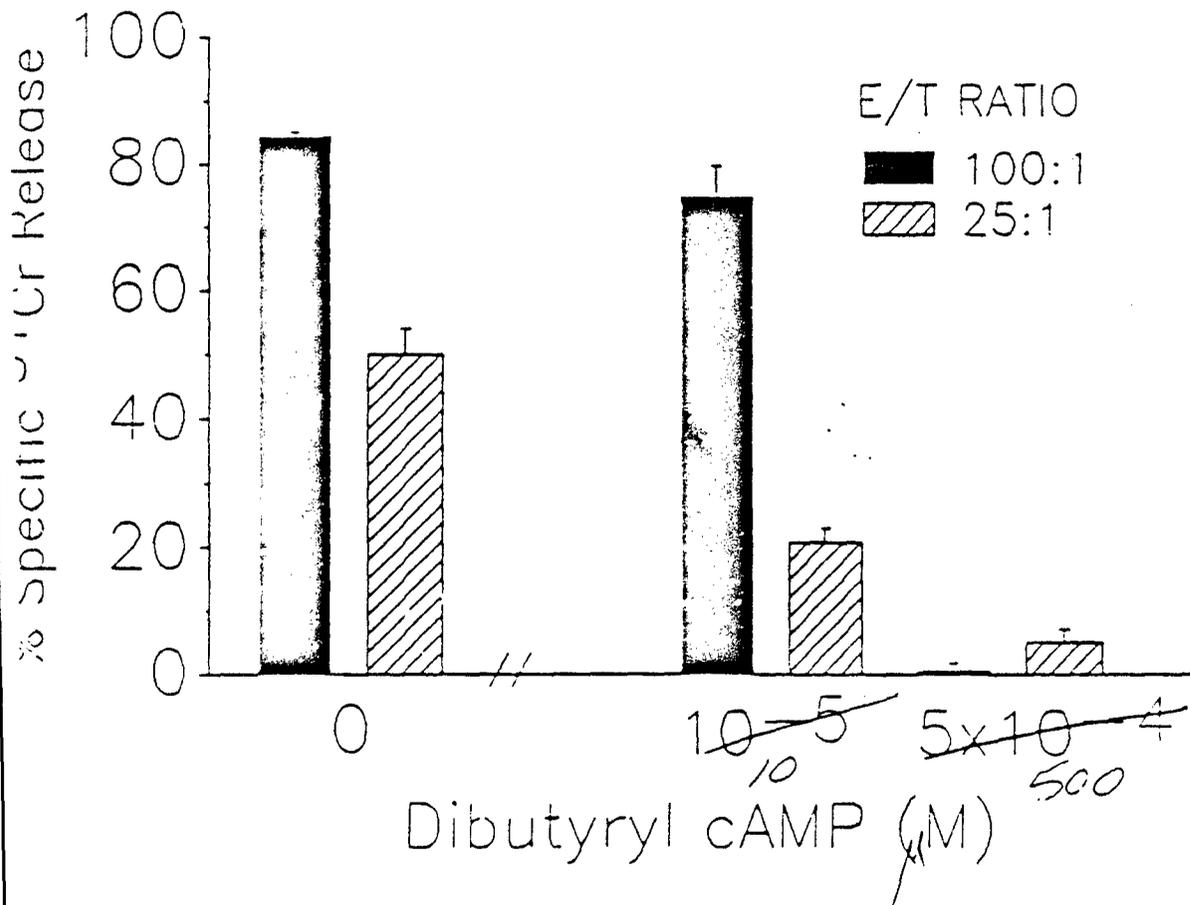
Figure 7. Effect of Propranolol on Norepinephrine Inhibition of the Generation of Anti-MOPC-315 Cytotoxicity by Spleen Cells from Melphalin-cured MOPC-315 Tumor Bearers. Spleen cells from melphalin-cured MOPC-315 tumor bearing BALB/c mice were incubated with propranolol for 30 minutes prior to addition of norepinephrine at 100  $\mu$ M (closed triangle, dashed line) and mitomycin-C-treated MOPC-315 cells.

Controls (*closed circle, solid line*) consisted of cells incubated with mitomycin-C-treated MOPC-315 cells in the absence of norepinephrine. After 5 days, the in vitro immunized cells were evaluated for their lytic activity. Data shown are for an effector/target cell ratio of 100:1. Data are from a representative experiment of two experiments. Where error bars are not shown, the error bars are smaller than the symbol.



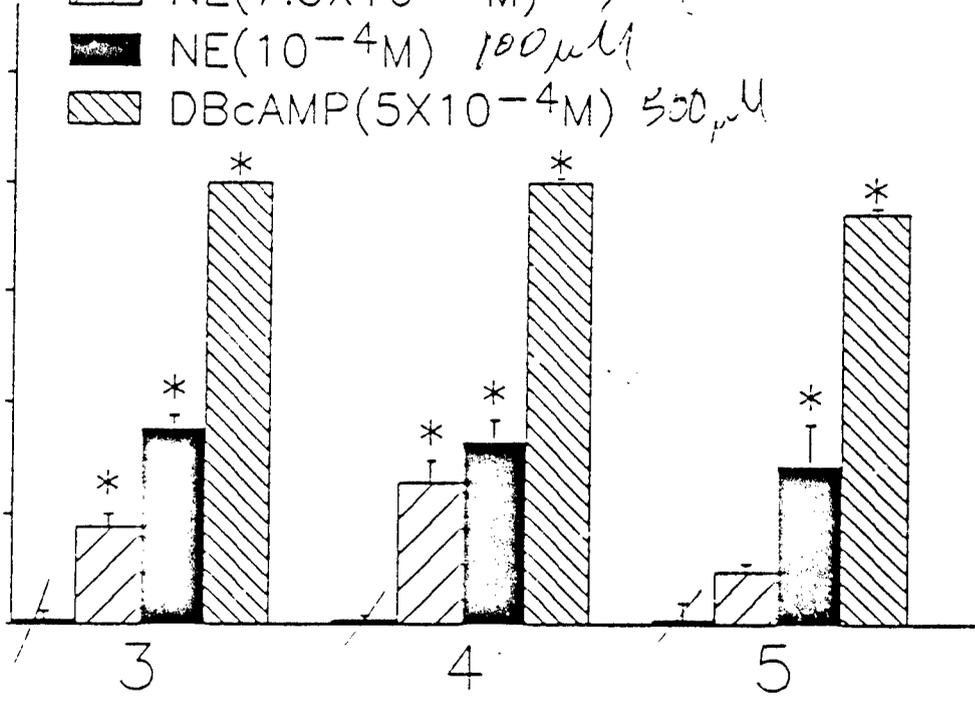


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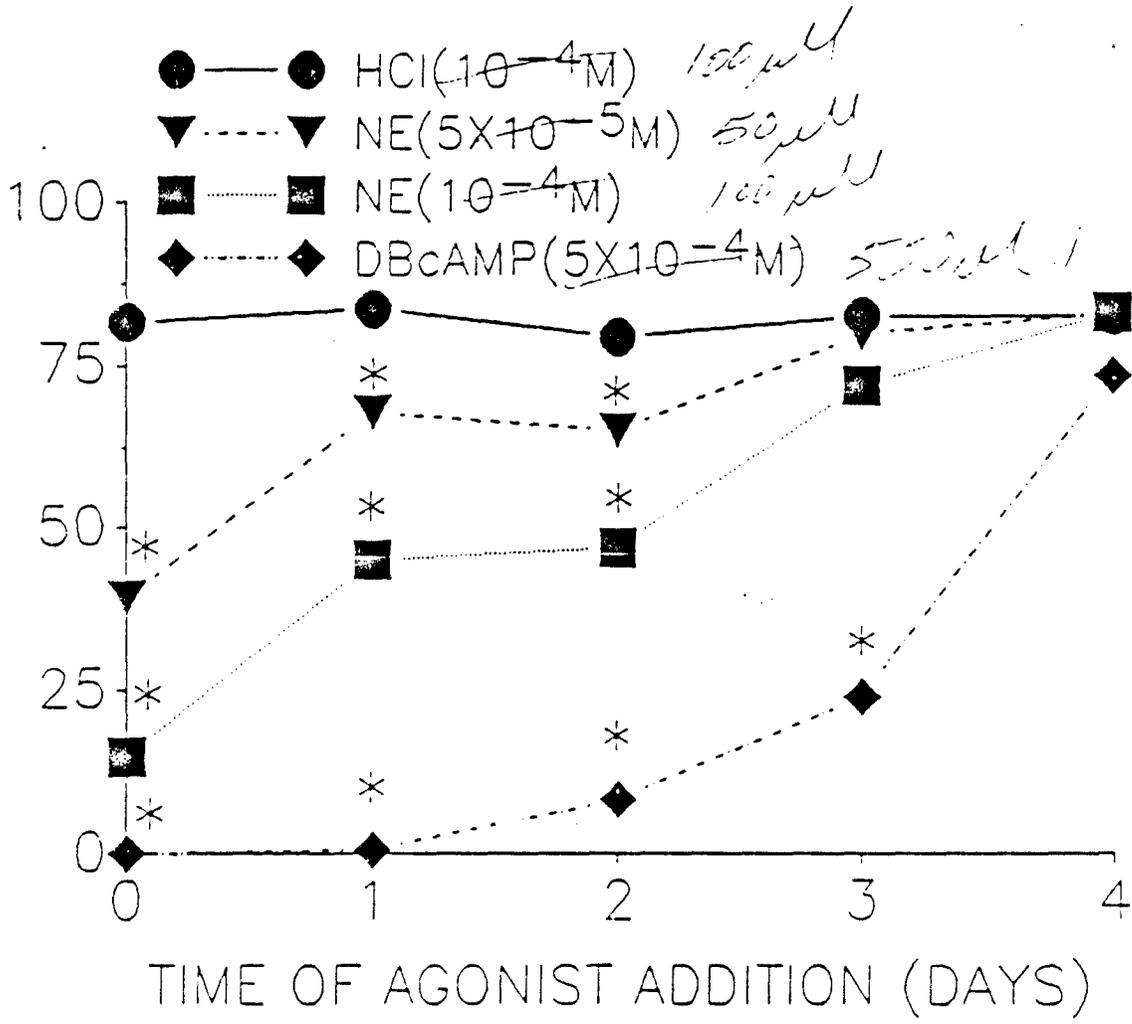
% Inhibition of MOPC-315-Stimulated anti-MOPC-315 Cytotoxicity

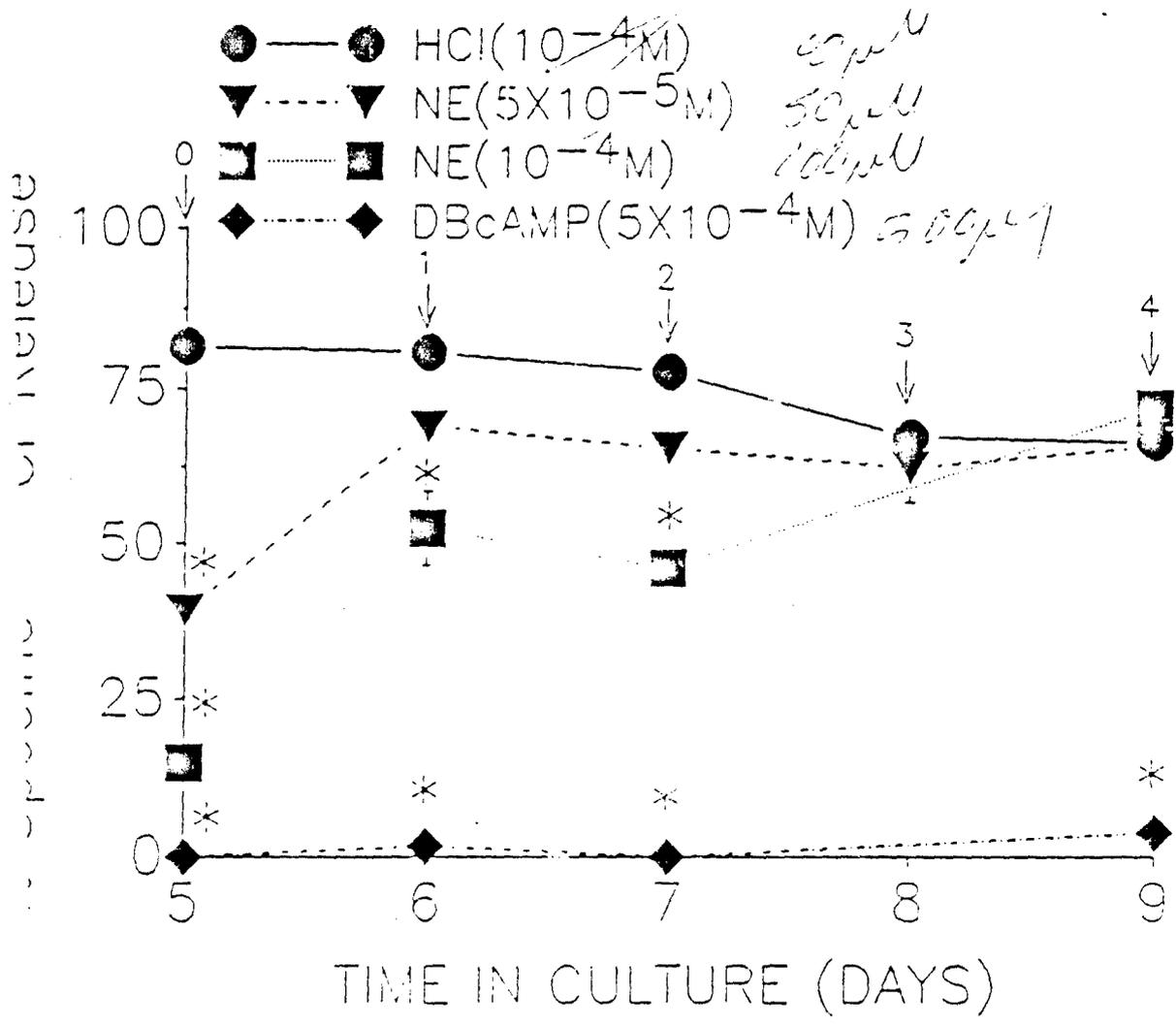
-  HCl ( $10^{-4}M$ ) 100  $\mu$ l
-  NE ( $7.5 \times 10^{-5}M$ ) 75  $\mu$ l
-  NE ( $10^{-4}M$ ) 100  $\mu$ l
-  DBcAMP ( $5 \times 10^{-4}M$ ) 500  $\mu$ l



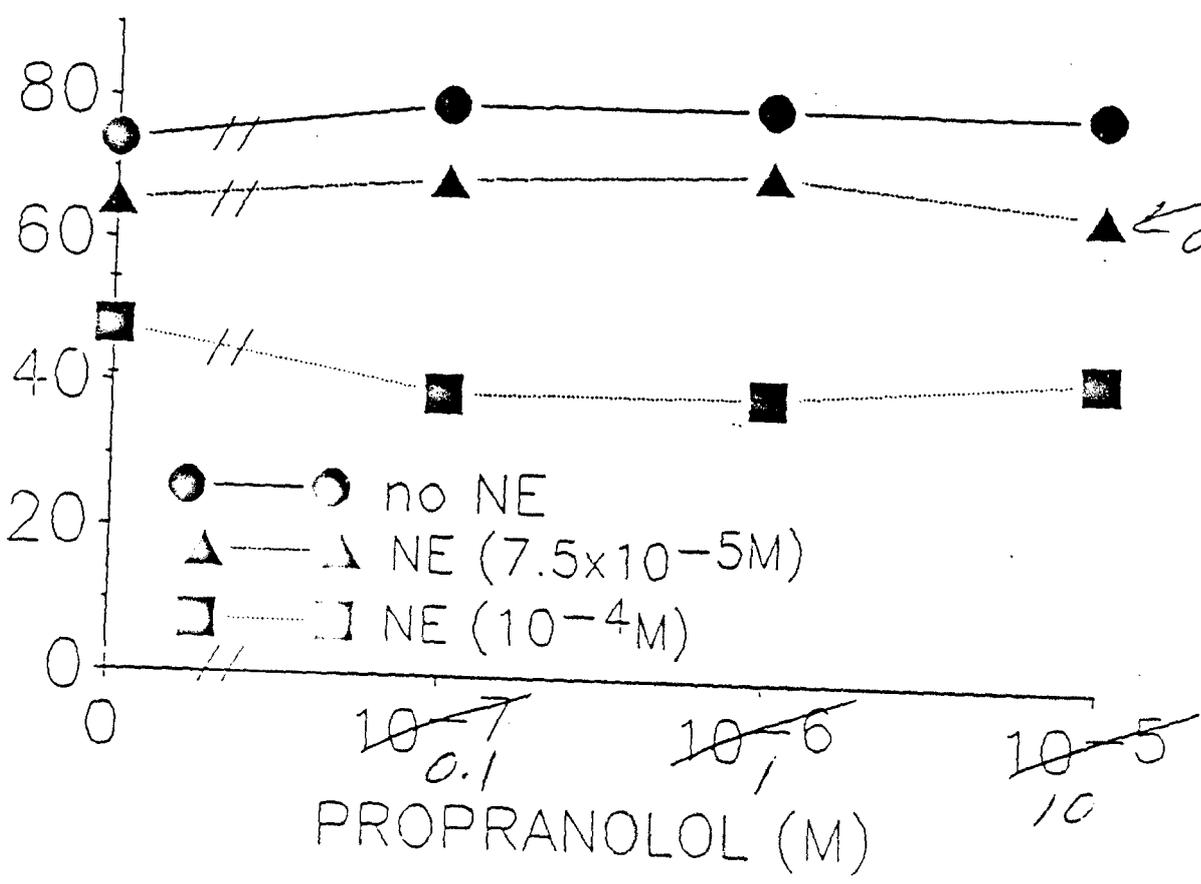
TIME IN CULTURE (DAYS)

ASPIRIN IN %





% Specific <sup>51</sup>Cr Release



● —● no NE  
▲ —▲ NE (7.5x10<sup>-5</sup>M)  
■ —■ NE (10<sup>-4</sup>M)

PROPRANOLOL (M)