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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION (U)			1b. RESTRICTIVE MARKINGS NA		
2a. SECURITY CLASSIFICATION AUTHORITY NA			3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution Unlimited		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE NA			5. MONITORING ORGANIZATION REPORT NUMBER(S) NA		
4. PERFORMING ORGANIZATION REPORT NUMBER(S) Univ. of Illinois at Chicago			7a. NAME OF MONITORING ORGANIZATION Office of Naval Research		
6a. NAME OF PERFORMING ORGANIZATION Univ. of Illinois at Chicago		6b. OFFICE SYMBOL (If applicable) NA		7b. ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000	
6c. ADDRESS (City, State, and ZIP Code) Univ. of Illinois at Chicago Periodontal Research Center P.O. Box 6998 Chicago, IL 60680		8b. OFFICE SYMBOL (If applicable) ONR		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER N00014-87-K-0027	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Office of Naval Research		8c. ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000		10. SOURCE OF FUNDING NUMBERS	
				PROGRAM ELEMENT NO 61153N	PROJECT NO RR04108
				TASK NO 4415716	WORK UNIT ACCESSION NO
11. TITLE (Include Security Classification) (U) Interactions of Neuromodulators with cells of the immune system.					
12. PERSONAL AUTHOR(S) Chambers, Donald A.					
13a. TYPE OF REPORT Annual		13b. TIME COVERED FROM 11/1/86 TO 10/31/87		14. DATE OF REPORT (Year, Month, Day) 8/5/88	
15. PAGE COUNT 20					
16. SUPPLEMENTARY NOTATION Keywords on file					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD 08	GROUP	SUB-GROUP	behavioral immunology; neural-immune interaction; neurotransmitters; neuropeptides; T and B lymphocytes; cell surface molecules; biologic response modifiers; (continued)		
19. ABSTRACT (Continue on reverse if necessary and identify by block number) The research program is designed to explore the effects of norepinephrine (NE) and other neuromodulators on non-neuronal cells. During the past year significant progress has been made in four inter-related areas: 1) The effects of norepinephrine on mitogen-stimulated spleen lymphocytes, thymocytes, nude mouse lymphocytes and Balb/c T cell lymphosarcoma cells (S49 cells) has been examined in a serum-free culture system. Norepinephrine has been shown to mediate inhibition of both T & B cell activation, probably through different mechanisms which may involve cyclic AMP in the T cell but not in the B cell. 2) NE also inhibits IL2 generated lymphocyte activation in serum-free culture, suggesting that the mechanisms of NE-mediated IL2 inhibition is distal to ConA binding and may also involve IL2 receptor regulation. 3) In contrast to its effects on immune cells, NE mediates keratinocyte mitogenesis. 4) NE appears to down regulate the appearance of thyl protein on the surface of lymphocytes, thymocytes and keratinocytes. parallel experiments with cAMP show a similar effect of cAMP-mediated thyl (continued)					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION (U)		
22a. NAME OF RESPONSIBLE INDIVIDUAL Dr. J.A. Maide			22b. TELEPHONE (Include Area Code) 202 696-4055		22c. OFFICE SYMBOL ONR

DD Form 1473, JUN 86

Previous editions are obsolete

SECURITY CLASSIFICATION OF THIS PAGE

S/N 0102-LF-014-6603

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lymphocyte activation, Thy-1 protein, cyclic nucleotides

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→ protein down regulation, suggesting that cAMP could be mechanistically involved with thyl gene expression. These observations supply the focus for work to be done in year two, directed towards understanding the mechanisms of the NE-mediated effects.

Report as: ... DD473 1A

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I. Introduction

The objectives of this proposal are to define basic parameters and directions for the study of neurotransmitters with cells of the immune system, using previously defined serum-free systems to examine biochemical, physiologic and specific responses of subpopulations of immune cells to neurotransmitters. During the past year, the major goal was to study the effects of neurotransmitters on spleen lymphocyte activation by both ConA- and IL-2-dependent systems and to subfractionate the complex spleen system into more homogeneous subpopulations in an attempt to determine the specific cellular responses. Progress has been made in four interrelated research areas:

- (1) We have utilized a serum-free lymphocyte activation system to study the effect of norepinephrine (NE), NE agonists and antagonists on mitogen-stimulated lymphocyte activation on lymphocytes derived from normal and nude mouse spleens, normal mouse thymocytes and a murine T-cell line (S49 lymphosarcoma cells).
- (2) We have defined a serum-free murine lymphocyte system that responds to IL-2 and examined the effects of NE and cyclic AMP on this system.
- (3) We have examined the effects of NE on mouse keratinocytes in culture.

- (4) We have begun the study of the effects of NE and cyclic AMP on Thy-1 protein expression in murine lymphocytes and keratinocytes.

II. Progress Report

A. Effects of NE and related molecules on mitogen-activated serum-free murine lymphocytes

In this series of experiments, lymphocytes were incubated in serum-free medium in the presence of the mitogens ConA and NE. 10^{-4} M NE maximally inhibited lymphocyte activation (Figure 1). Equivalent dose responses were found for NE in both spleen lymphocytes and thymocytes. A time course study revealed that the inhibition response modulated by NE occurred within the first 24 hours after ConA addition (Figure 2). Since DNA synthesis in these cultures does not begin to increase until about 30 hours, the major NE effect seems to be in G1 of the cell cycle. Addition of DBcAMP, 8BrcAMP, cholera toxin, forskolin or dopamine (10^{-4} M) inhibited DNA synthesis by ConA-activated lymphocytes; however, 5 hydroxytryptamine (5HT) had no effect (Figure 3). These results suggested that the NE effect could be modulated, in part, through the β -adrenergic receptor. In addition, α -adrenergic agonists were found to have no inhibiting effect (Table 1). Since ConA is a T-cell mitogen, the inhibiting effects of NE in this spleen lymphocyte preparation is most likely T-cell dependent. Consistent with this hypothesis is the fact that (1) NE is a potent inhibitor of thymocytes (Figure 4) and (2) in preliminary experiments, NE is also inhibitory to the S49 BALB/c lymphosarcoma T-cell line. This latter data could be of additional significance because it relates to a homogeneous lymphocyte population which, unlike primary spleen cell

preparations, is devoid of macrophages. This data suggests that, although NE could have cellular targets other than lymphocytes, it has direct effects on cells of T lineage.

In parallel experiments utilizing the T-cell-independent, B-cell-mitogen LPS on mouse spleen lymphocytes, similar results were obtained with similar concentrations of NE, revealing that NE was inhibitory to B-cell activation within 24 hours of LPS addition (Figure 5). However, unlike the situation with NE and ConA activation, cAMP and related molecules had little, if any, inhibitory activity in the presence of B-cells (Figure 6). Similar responses occurred when lymphocytes were obtained from T-cell mitogen-incompetent nude mouse spleens, revealing that NE could inhibit B-cells but cAMP could not (Figure 7). Thus, these results lead to the exciting conclusion that, although NE is inhibitory to both T- and B-lymphocytes, the NE-related T-cell inhibition may be mediated through a cAMP-dependent pathway, whereas NE-B-cell inhibition is cAMP-independent. To our knowledge, this is the first report of a T-cell-associated, NE-modulated, cAMP-dependent inhibitory system and a B-cell-associated, NE-modulated, cAMP-independent inhibitory pathway.

Experiments to be performed in the coming year will be designed to elucidate potential mechanisms through which NE inhibits T- and B-cell activation.

B. Interleukin-2 (IL2) dependent serum-free lymphocyte activation system

In the past year, in order to study the effect of the T-cell-activating lymphokine IL-2, we developed a serum-free lymphocyte activation system which responded to IL-2. This system has the potential of allowing us to study activation events distal to the addition of ConA. In previous experiments we reported that lymphocyte inhibition by cAMP occurred within the first 12 hours after ConA addition. The addition of cAMP at later periods resulted in a markedly decreased inhibitory effect. Thus, a cAMP-modulated inhibitory event prevents lymphocyte activation early in the lymphocyte's proliferative program. The discovery of IL-2 and delineation of its temporal effects during lymphocyte activation suggested that cAMP could inhibit the synthesis and/or release of IL-2. If such were the case, cAMP should have no effect in the presence of excess IL-2. To test this hypothesis, we utilized the IL-2-dependent activation system and examined the effect of added DBcAMP. To our surprise, cAMP was markedly more inhibitory to both ConA-stimulated and IL-2-stimulated lymphocyte activation (Figure 8). Parallel experiments employing NE in place of DBcAMP revealed that NE could also inhibit IL-2-mediated activation (Figure 9). These experiments suggest that both NE and cAMP can modulate lymphokine activity. Such activity could be mechanistically distal to the IL-2 effect or could relate to the regulation of the IL-2 receptor. These possibilities will be the focus of experiments in the coming year.

C. Effect of NE on epidermal cells

One of the major thrusts of this research program is to examine the effects of neuromodulators on non-neuronal cells. This laboratory is well equipped to undertake such studies since we also are involved in a large research effort studying epidermal homeostasis, both in vivo and in vitro. In addition, new understandings of skin have produced analogies between the epidermis and the immune system. Both systems are fundamental components in protection of the organism. In fact, most recently, we and others have been studying elements common to both systems, leading to an emerging concept of skin as a "peripheral immune organ."

Past work in this and other laboratories has shown that cAMP is an epidermal mitogen. These results suggested to us that perhaps NE would have mitogenic effects on epidermal cells, perhaps involving a β -adrenergic-mediated, cAMP-responsive pathway in these cells. Figure 10 shows that, indeed, addition of NE to epidermal cultures leads to an enhanced mitogenic response. In addition, these observations reveal that NE acts in tissue-specific ways; namely, it augments mitogenesis in epidermal cells and inhibits T-cell activation in the immune system. The fundamental mechanism underlying these observations will be explored in the coming year.

D. Regulation of Thy-1 protein expression in epidermal keratinocytes and lymphocytes

Thy-1 protein is a cell surface protein (member of the immunoglobulin supergene family) expressed by lymphocytes, nerve and brain cells and fibroblasts. Recently, we have discovered that at least two populations express comparable levels of Thy-1 protein in the epidermis--a bone marrow derived dendritic cell (now termed the Thy-1 dendritic cell [Thy-1 DEC]) and a minority population of keratinocytes. The functional roles of these cells awaits definition. However, we have recently observed that culturing lymphocytes or epidermal cells in the presence of cAMP or NE leads to a diminished presence of cell surface Thy-1 in both systems. (Figures 11 and Table 2). In the case of lymphocytes, diminished presence of Thy-1 protein correlates with a parallel inhibition of response to ConA stimulation. These results are very exciting to us because they supply the framework for further direct study of NE modulation of the Thy-1 gene in both immune cells and cells of epithelial origin. In addition, this system may further allow us to develop a specific molecular biological approach of studying the regulation of a gene product (Thy-1 protein) that may have significance in signal transduction in both the immune system and the skin.

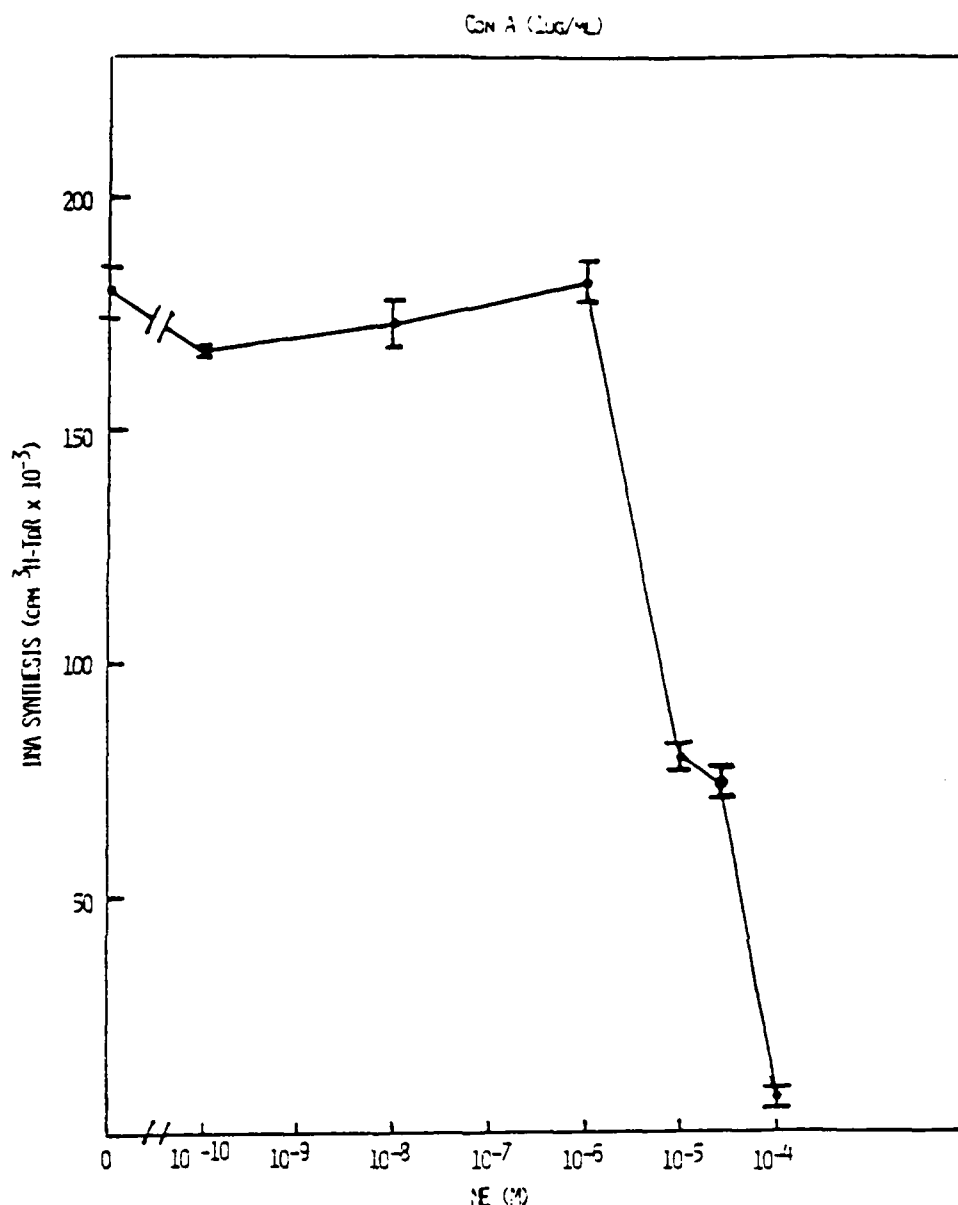


Figure 1. Effect of increasing NE concentration on BALB/c mouse spleen lymphocyte DNA synthesis. Addition of NE in ConA-stimulated DNA synthesis demonstrates maximal effect at 10^{-4}M .

BALB/c mouse lymphocytes (5×10^6 cells/ml, pooled cells from 4-5 males, 6-10 weeks of age) were cultured in serum-free RPMI-1640 containing $5 \times 10^{-5}\text{M}$ B_2 mercaptoethanol, penicillin and streptomycin. ConA ($1\mu\text{g/ml}$) and norepinephrine (10^{-4}M to 10^{-10}M) were added at culture initiation. Cells were incubated in 96-well microtiter plates at 37°C in a humid 5% CO_2 atmosphere. At 44 h cells were pulsed with (^3H)TdR ($1\mu\text{Ci}$, 20Ci/mM) for 4 h. (^3H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

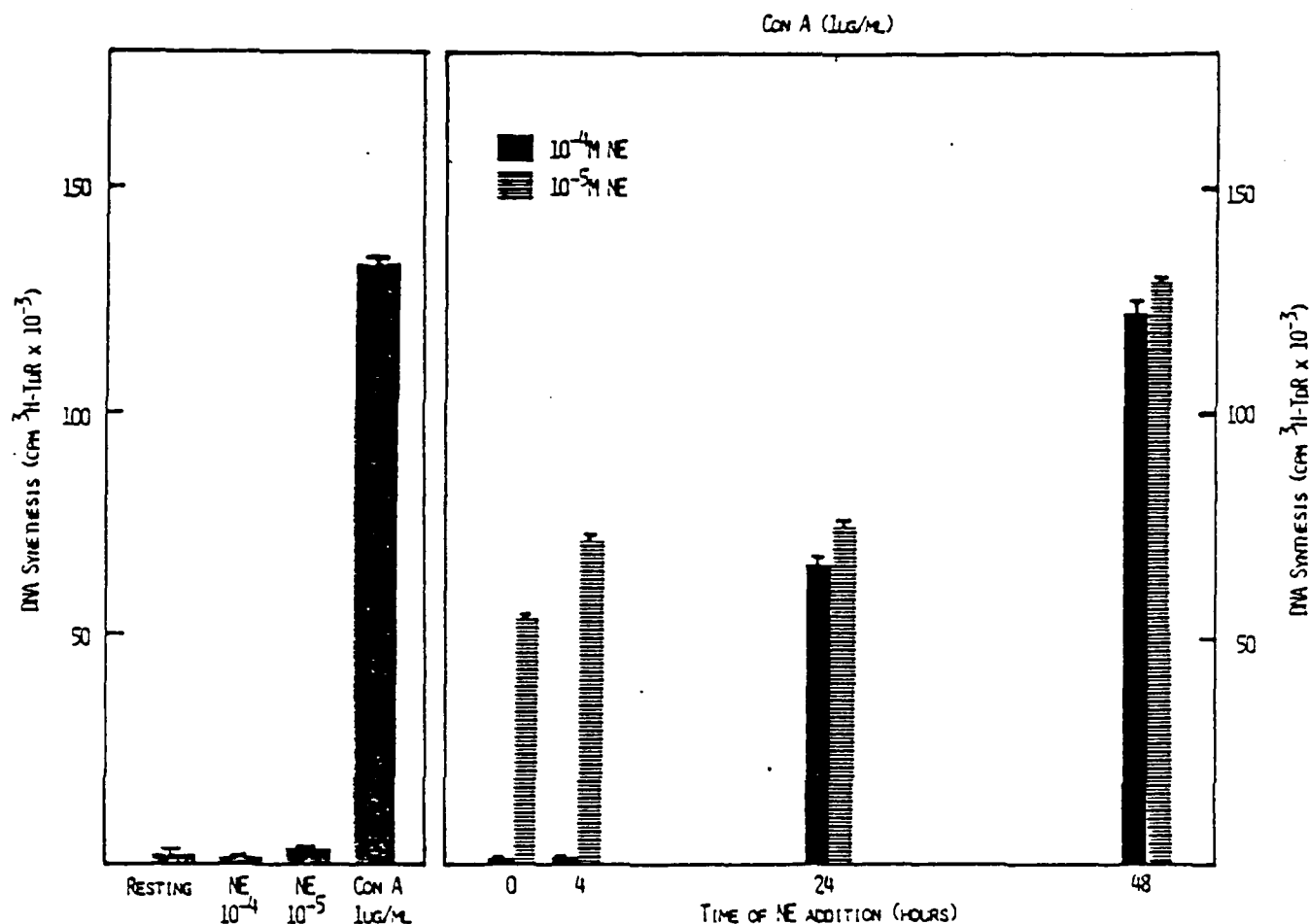


Figure 2. Effect of varying time of NE addition on DNA synthesis by ConA-stimulated BALB/c mouse spleen lymphocytes revealed maximal effect by 24 hours of culture.

BALB/c mouse lymphocytes (5×10^{-6} cells/ml, pooled cells from 4-5 males, 6-10 weeks of age) were cultured in serum-free RPMI-1640 containing $5 \times 10^{-5}\text{M}$ B_2 mercaptoethanol, penicillin and streptomycin. ConA ($1\mu\text{g/ml}$) and norepinephrine (10^{-4}M to 10^{-5}M) were added at times indicated. Cells were incubated in 96-well microtiter plates at 37°C in a humid 5% CO_2 atmosphere. At 44 h cells were pulsed with (^3H)TdR ($1\mu\text{Ci}$, 20Ci/mM) for 4 h. (^3H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

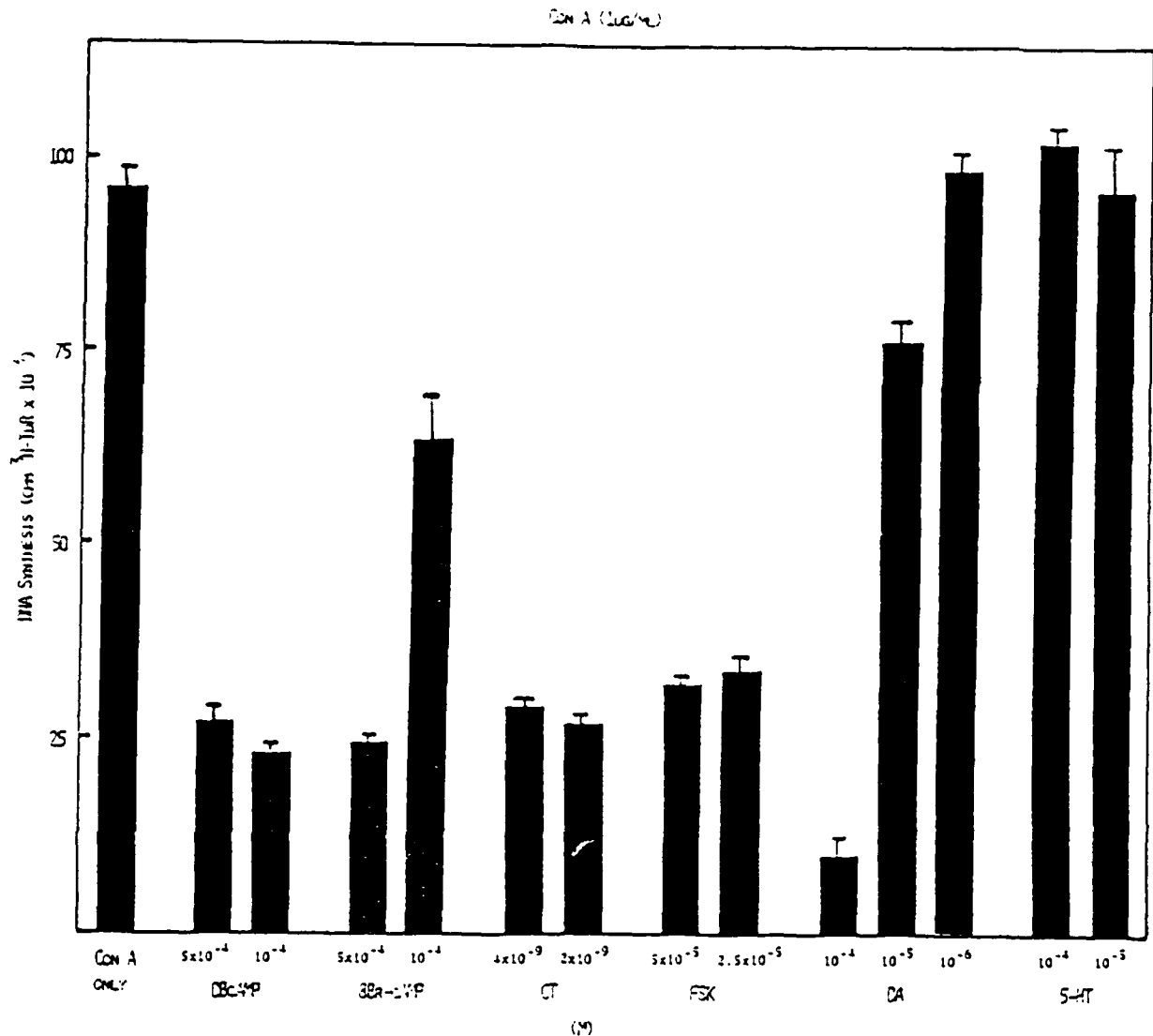


Figure 3. Cyclic AMP, cholera toxin and forskolin and dopamine can inhibit ConA-stimulated DNA synthesis by BALB/c spleen lymphocytes in contrast to 5-hydroxytryptamine.

BALB/c mouse lymphocytes (5×10^{-6} cells/ml, pooled cells from 4-5 males, 6-10 weeks of age) were cultured in serum-free RPMI-1640 containing 5×10^{-5} M B_2 mercaptoethanol, penicillin and streptomycin. ConA (1 $\mu\text{g}/\text{ml}$), DBcAMP, 8Br-cAMP, cholera toxin (CT), forskolin (FSK), dopamine (DA) and 5-hydroxytryptamine (5-HT) were added at culture initiation at the concentrations indicated. Cells were incubated in 96-well microtiter plates at 37°C in a humid 5% CO_2 atmosphere. At 44 h cells were pulsed with (^3H)TdR (1 μCi , 20 Ci/mmol) for 4 h. (^3H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

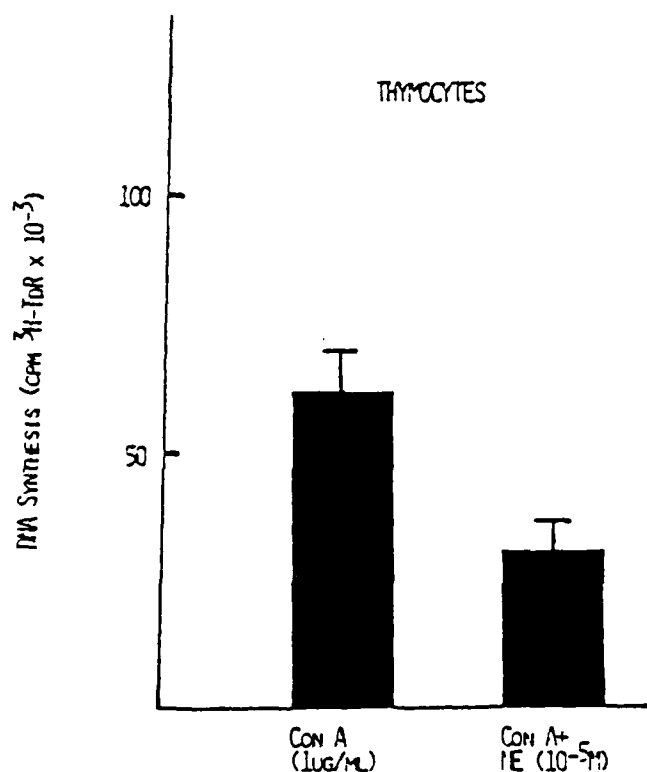


Figure 4. Addition of NE suppresses ConA-induced DNA synthesis in BALB/c mouse thymocytes.

BALB/c mouse thymocytes (5×10^{-6} cells/ml, pooled cells from 4-5 males, 6-10 weeks of age) were cultured in serum-free RPMI-1640 containing $5 \times 10^{-5}\text{M}$ B_2 mercaptoethanol, penicillin and streptomycin. ConA (1ug/ml) and norepinephrine (10^{-5}M) were added at culture initiation. Cells were incubated in 96-well microtiter plates at 37°C in a humid 5% CO_2 atmosphere. At 44 h cells were pulsed with (^3H)TdR (1μCi, 20C/mM) for 4 h. (^3H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

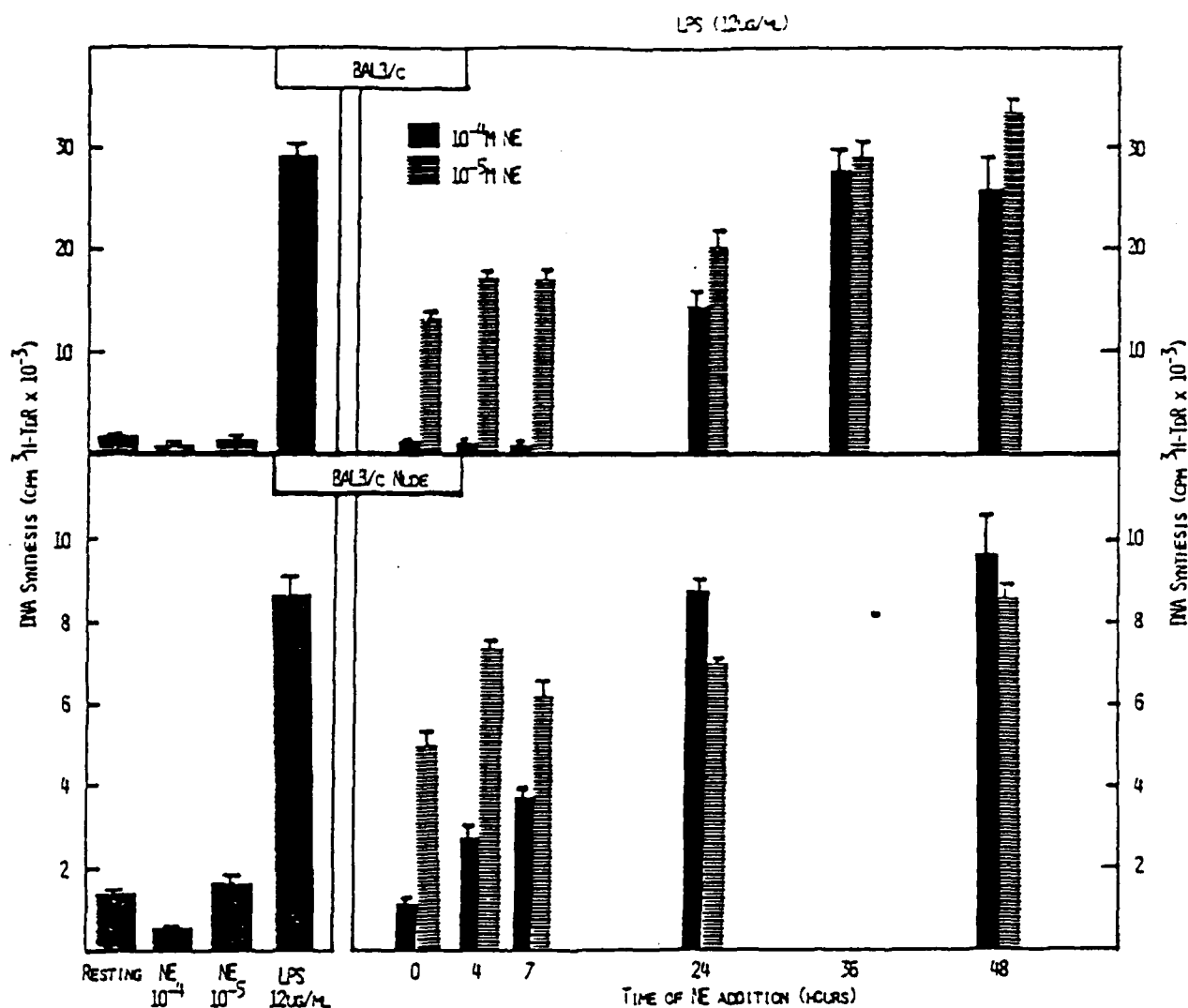


Figure 5. Addition of NE to normal or athymic (nude) mouse spleen cells at varying times after stimulation with the B-cell mitogen lipopolysaccharide reveals maximal suppression of DNA synthesis occurs within 24 hours of LPS addition.

BALB/c normal or athymic (nude) mouse lymphocytes (5×10^{-6} cells/ml, pooled cells from 4-5 males, 6-10 weeks of age) were cultured in serum-free RPMI-1640 containing $5 \times 10^{-5}\text{M}$ B_2 mercaptoethanol, penicillin and streptomycin. LPS (12 $\mu\text{g}/\text{ml}$) was added at culture initiation and norepinephrine (10^{-4}M or 10^{-5}M) were added at culture initiation. Cells were incubated in 96-well microtiter plates at 37°C in a humid 5% CO_2 atmosphere. At 44 h cells were pulsed with (^3H)TdR (1 μCi , 20C/mM) for 4 h. (^3H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

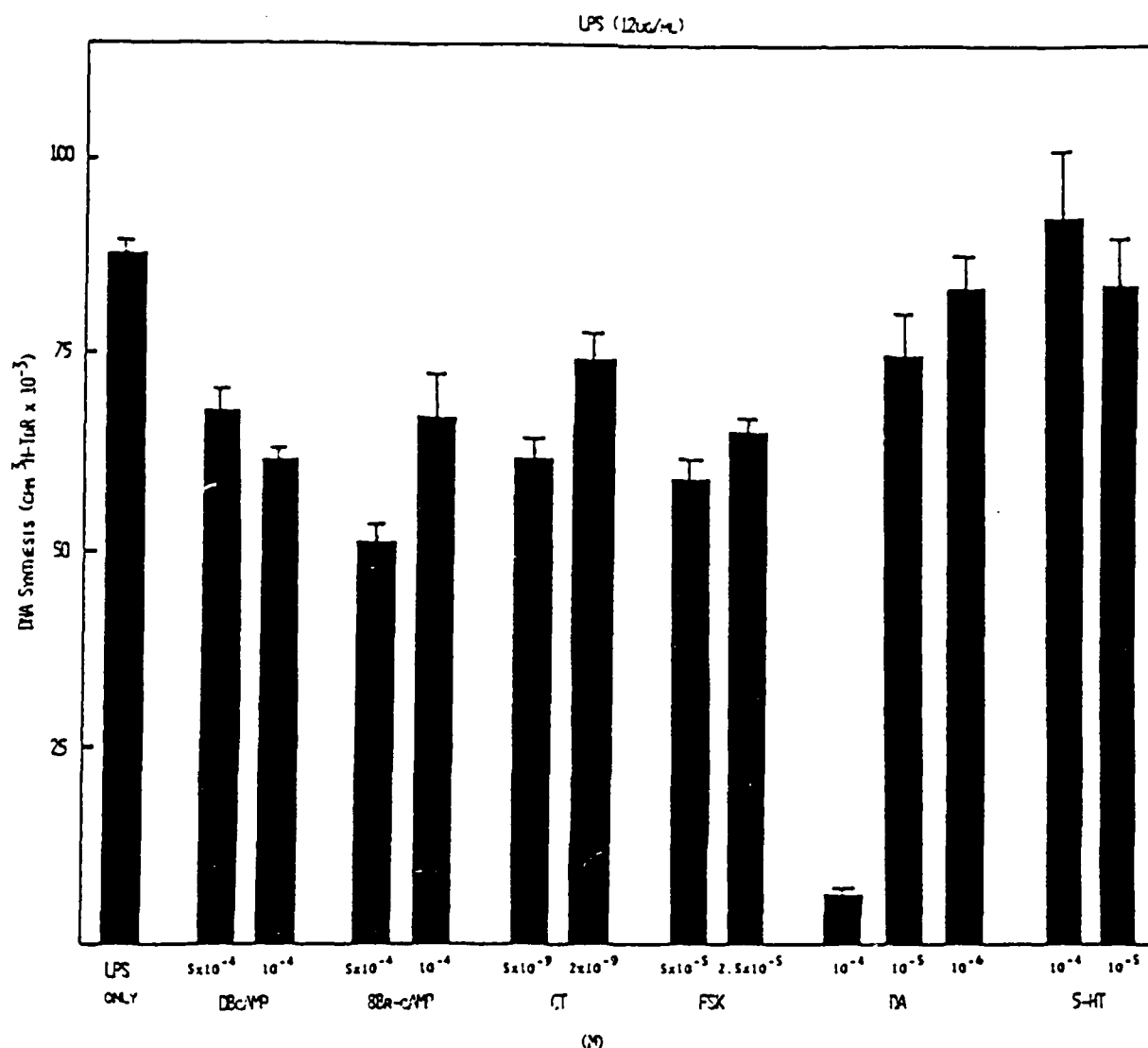


Figure 6. Cyclic AMP, cholera toxin, forskolin and 5-hydroxytryptamine had no effect on LPS-stimulated DNA synthesis by BALB/c spleen lymphocytes.

BALB/c mouse lymphocytes (5×10^{-6} cells/ml, pooled cells from 4-5 males, 6-10 weeks of age) were cultured in serum-free RPMI-1640 containing 5×10^{-5} M B_2 mercaptoethanol, penicillin and streptomycin. LPS (12 μ g/ml), DBcAMP, 8Br-cAMP, cholera toxin (CT), forskolin (FSK), dopamine (DA) and 5-hydroxytryptamine (5-HT) were added at culture initiation at the concentrations indicated. Cells were incubated in 96-well microtiter plates at 37°C in a humid 5% CO_2 atmosphere. At 44 h cells were pulsed with (3 H)TdR (1 μ Ci, 20C/mM) for 4 h. (3 H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

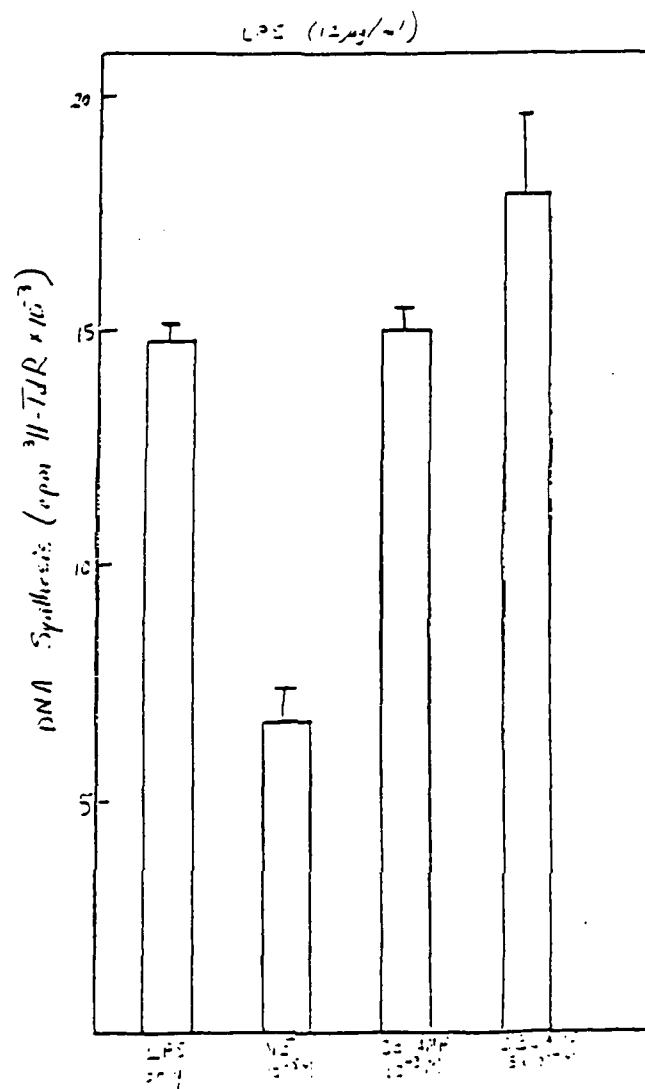


Figure 7. cAMP did not inhibit LPS-stimulated DNA synthesis by athymic (nude) mouse lymphocytes.

BALB/c nude mouse lymphocytes (pooled cells from 4-5 males, 6-10 weeks of age) were cultured in serum-free RPMI-1640 containing 5×10^{-5} M B_2 mercaptoethanol, penicillin and streptomycin. LPS (12 μ g/ml) and cAMP or norepinephrine were added at culture initiation. Cells were incubated in 96-well microtiter plates at 37°C in a humid 5% CO_2 atmosphere. At 44 h cells were pulsed with (3 H)TdR (1 μ Ci, 200 nM) for 4 h. (3 H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

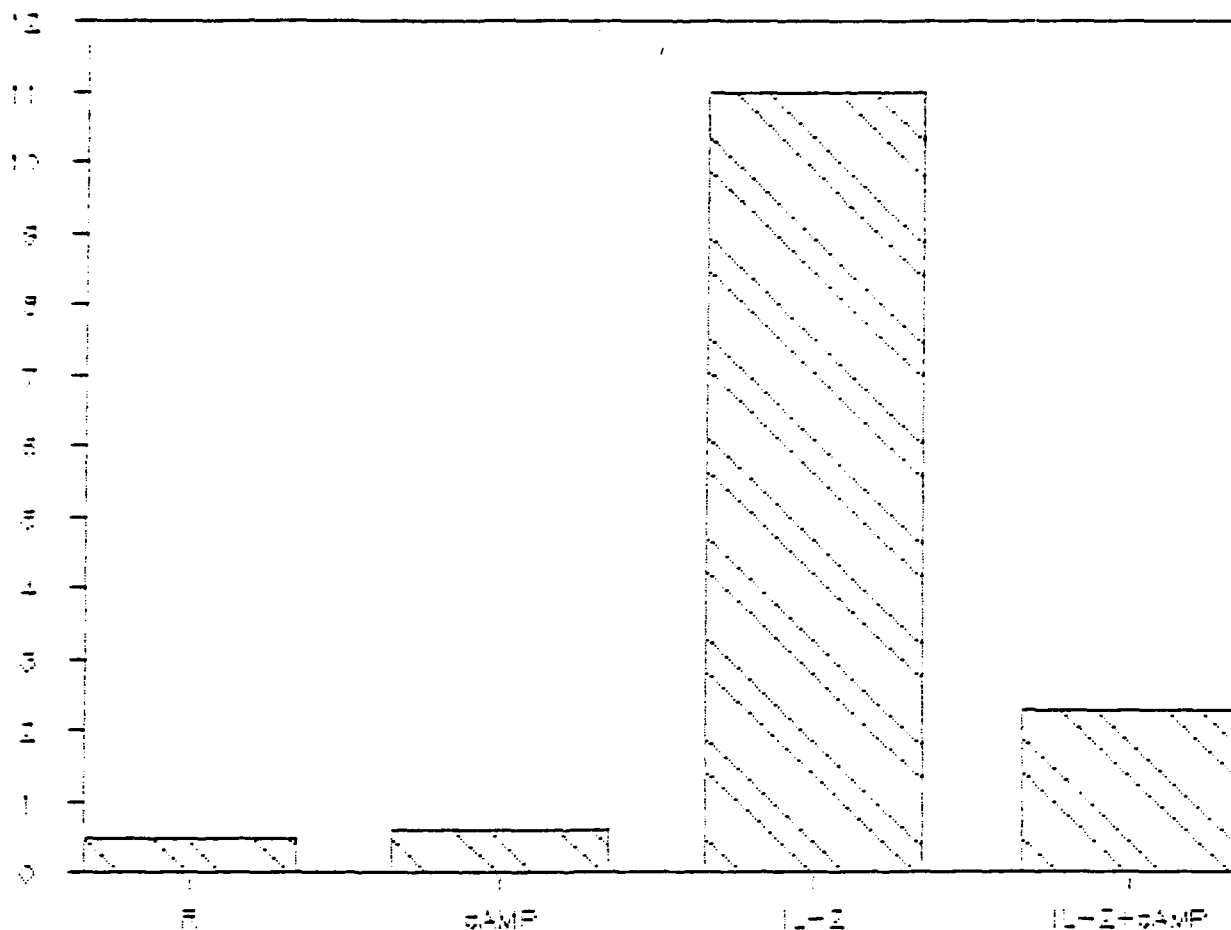


Figure 8. Cyclic AMP inhibited IL-2-stimulated spleen lymphocyte DNA synthesis.

BALB/c mouse lymphocytes (5×10^{-6} cells/ml, pooled cells from 4-5 males, 6-10 weeks of age) were cultured in serum-free RPMI-1640 containing 5×10^{-5} M B_2 mercaptoethanol, penicillin and streptomycin. DBcAMP (5×10^{-4} M) and IL-2 (50U/ml) were added at culture initiation. Cells were incubated in 96-well microtiter plates at 37°C in a humid 5% CO_2 atmosphere. At 44 h cells were pulsed with (^3H)TdR ($1\mu\text{Ci}$, $20\text{Ci}/\text{mM}$) for 4 h. (^3H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

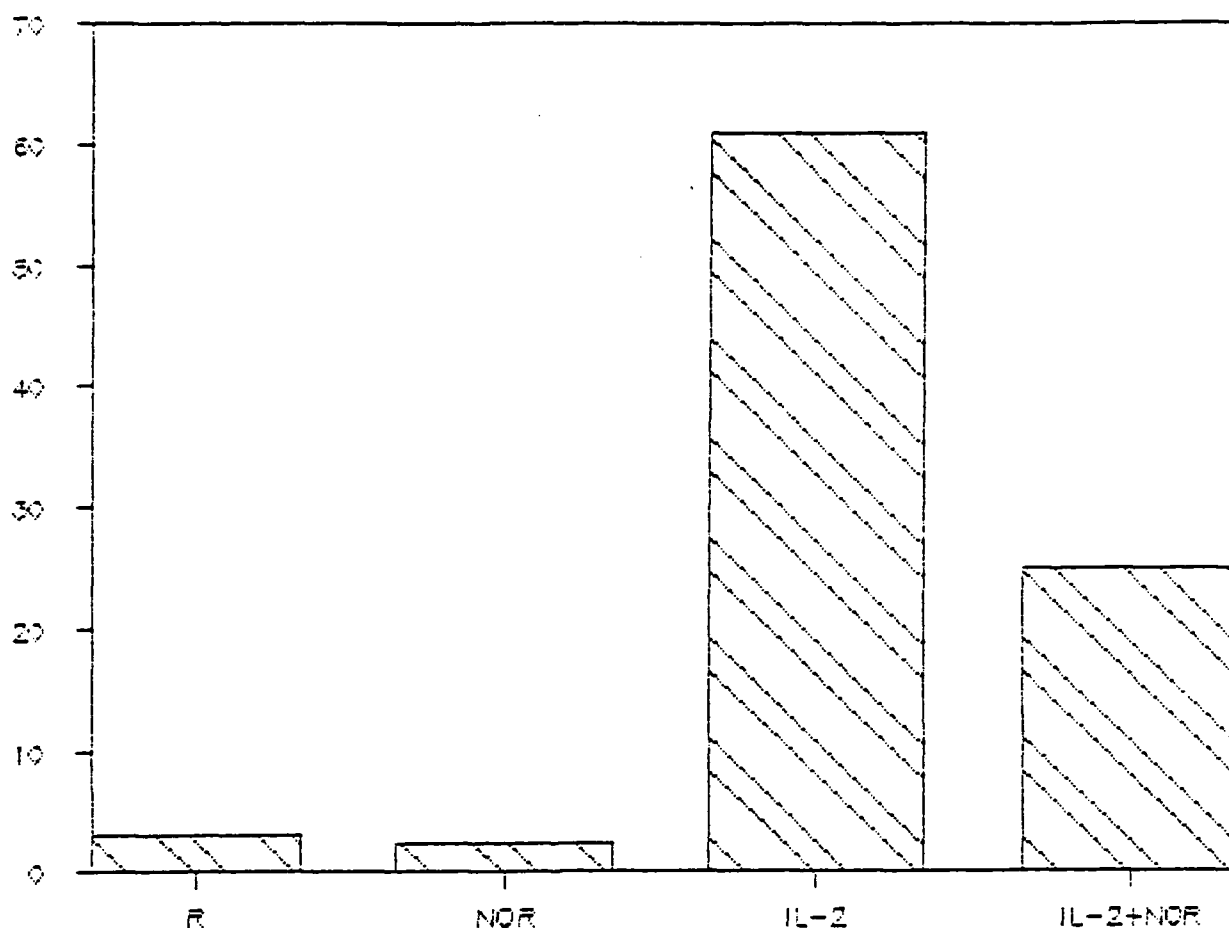


Figure 9. NE inhibited IL-2-stimulated spleen lymphocyte DNA synthesis.

BALB/c mouse lymphocytes (5×10^{-6} cells/ml, pooled cells from 4-5 males, 6-10 weeks of age) were cultured in serum-free RPMI-1640 containing 5×10^{-5} M B_2 mercaptoethanol, penicillin and streptomycin. IL-2 (50U/ml) and norepinephrine (10^{-4} M) were added at culture initiation at the concentrations indicated. Cells were incubated in 96-well microtiter plates at 37°C in a humid 5% CO_2 atmosphere. At 44 h cells were pulsed with (^3H)TdR (1 μCi , 20C/mM) for 4 h. (^3H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

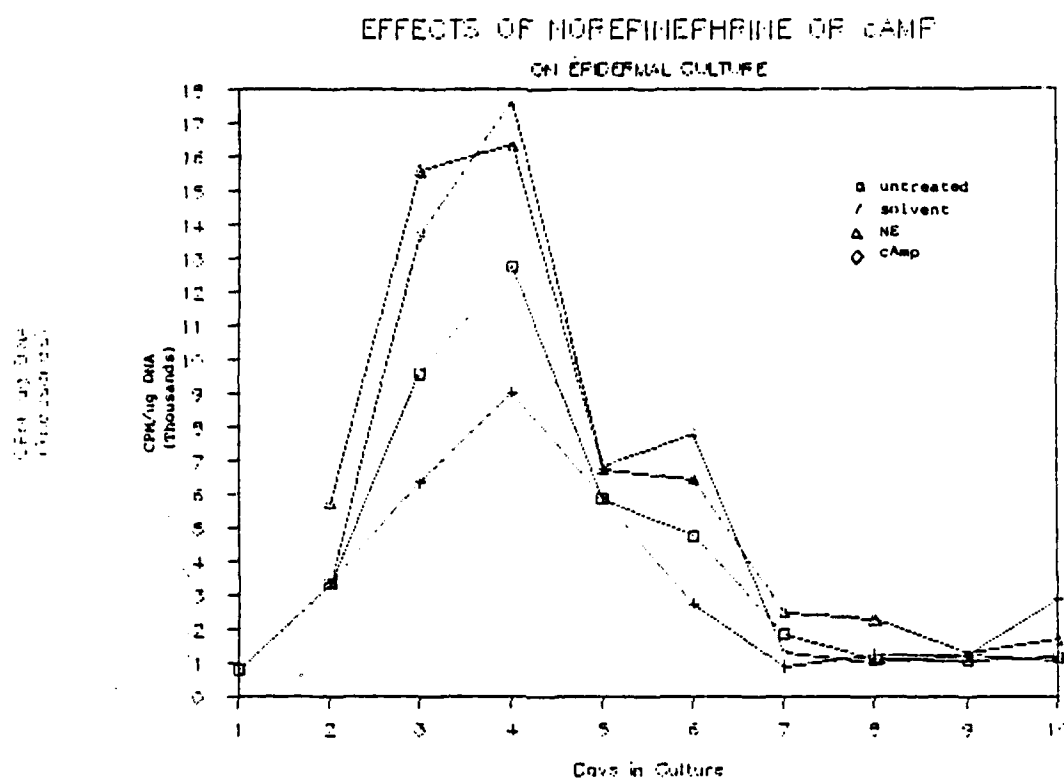


Figure 10. Addition of NE or cAMP lead to enhanced DNA synthesis by BALB/c mouse epidermal cells in culture.

Neonatal BALB/c mouse epidermal cells were obtained by trypsinization and centrifugation through a ficoll gradient. Cell suspensions (10^6 cells/ml) were incubated in RPMI-1640 containing 13% heat-inactivated fetal calf serum and antibiotics in a 5% CO_2 humid atmosphere at 32°C . NE (10^{-6}M) or cAMP (10^{-3}M) were added 18 h after culture initiation and replaced every 48 h at each media change. DNA synthesis was assayed by incorporation of (^3H)TdR during a 4 h pulse. Two representative culture dishes were pulsed each day with (^3H)TdR for 4 h. Acid-precipitable (^3H)DNA was assayed by liquid scintillation counting.

EFFECTS OF NOREPINEPHRINE OR CAMP ON THY-1 EXPRESSION BY EPIDERMAL CELLS

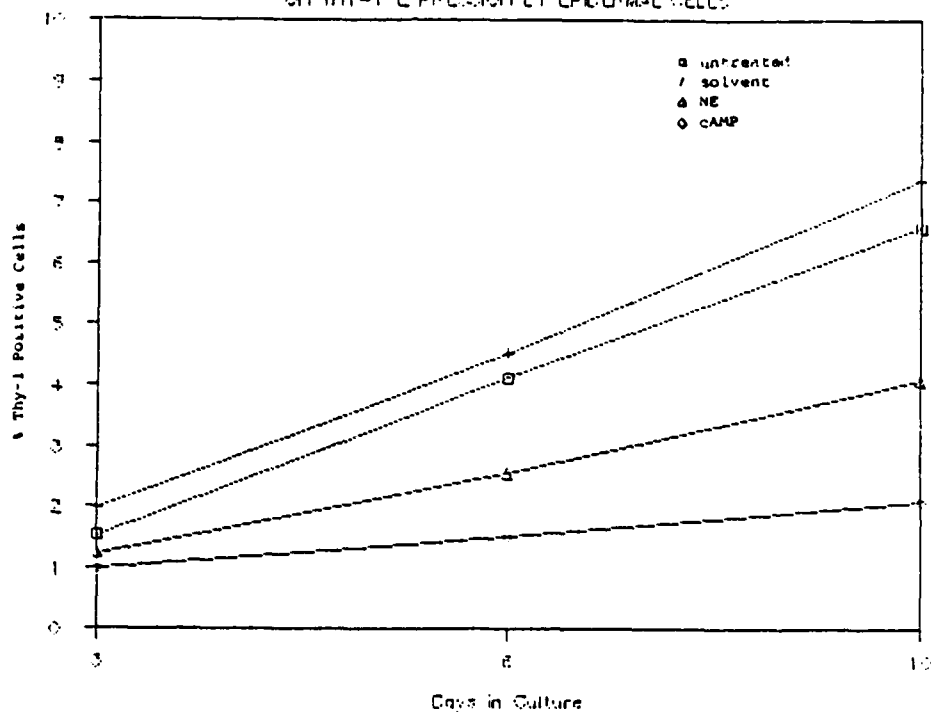


Figure 11. Thy-1 protein expression by neonatal BALB/c mouse epidermal cells in culture was diminished by addition of NE (10^{-6} M), cAMP (10^{-3} M) or cholera toxin (10^{-9} M). Epidermal cells were cultured as described in Figure 10, released into single cell suspension by brief incubation with EDTA and trypsin and prepared for fluorescence microscopy by standard methods. Data represents in excess of 1000 cells counted for each variable.

TABLE 1

EFFECTS OF α -ADRENERGIC RECEPTORS ON
ConA OR LPS STIMULATION OF BALB/c SPLEEN CELLS

Effect of Phenylephrine (α_1 agonist) on DNA Synthesis

	<u>DNA Synthesis (cpm ^3H-TdR)</u>
Resting	3,006 \pm 355
Phenylephrine (10^{-4}M)	2,514 \pm 210
Phenylephrine (10^{-5}M)	3,446 \pm 523
ConA	114,240 \pm 1614
ConA and Phenylephrine (10^{-4}M)	122,952 \pm 1160
ConA and Phenylephrine (10^{-5}M)	126,303 \pm 3655
LPS	77,118 \pm 1922
LPS and Phenylephrine (10^{-4}M)	73,684 \pm 1090
LPS and Phenylephrine (10^{-5}M)	72,927 \pm 1820

Effect of Clonidine (α_2 agonist) on DNA Synthesis

	<u>DNA Synthesis (cpm ^3H-TdR)</u>
Resting	1,744 \pm 227
Clonidine (10^{-4}M)	1,281 \pm 270
Clonidine (10^{-5}M)	1,693 \pm 260
ConA	66,802 \pm 6454
ConA and Clonidine (10^{-4}M)	50,576 \pm 2100
ConA and Clonidine (10^{-5}M)	49,190 \pm 2752
LPS	87,954 \pm 1367
LPS and Clonidine (10^{-5}M)	84,249 \pm 6888

BALB/c mouse lymphocytes (5×10^6 cells/ml, pooled cells from 4-5 males, 6-10 weeks of age) were cultured in serum-free RPMI-1640 containing $5 \times 10^{-5}\text{M}$ B_2 mercaptoethanol, penicillin and streptomycin. ConA ($1\mu\text{g/ml}$), DBcAMP, 8Br-cAMP, cholera toxin (CT), forskolin (FSK), dopamine (DA) and 5-hydroxytryptamine (5-HT) were added at culture initiation at the concentrations indicated. Cells were incubated in 96-well microtiter plates at 37°C in a humid 5% CO_2 atmosphere. At 44 h cells were pulsed with (^3H)TdR ($1\mu\text{Ci}$, 20Ci/mM) for 4 h. (^3H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

Table 2

Effect of Norepinephrine and cAMP on
Thy-1 Expression in BALB/c Lymphocytes

	<u>% Thy-1⁺ Lymphocytes</u>
Untreated	40
Norepinephrine(10^{-5} M)	29
Norepinephrine(10^{-4} M)	29
Dibutyryl cAMP(5×10^{-4} M)	22

Table 2. Thy-1 protein expression by BALB/c mouse spleen lymphocytes in culture diminished by addition of NE or cAMP.

Spleen lymphocytes were maintained in serum-free RPMI-1640 in the presence or absence of NE (10^{-4}) or cAMP (5×10^{-4} M) for 48 h at 37°C. Cells were then stained with a monoclonal anti-Thy-1.2 antibody and prepared for immunofluorescence microscopy by standard methods. Data represents in excess of 250 cells counted for each variable.

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