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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)					
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Block 18 continued from front
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.

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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.

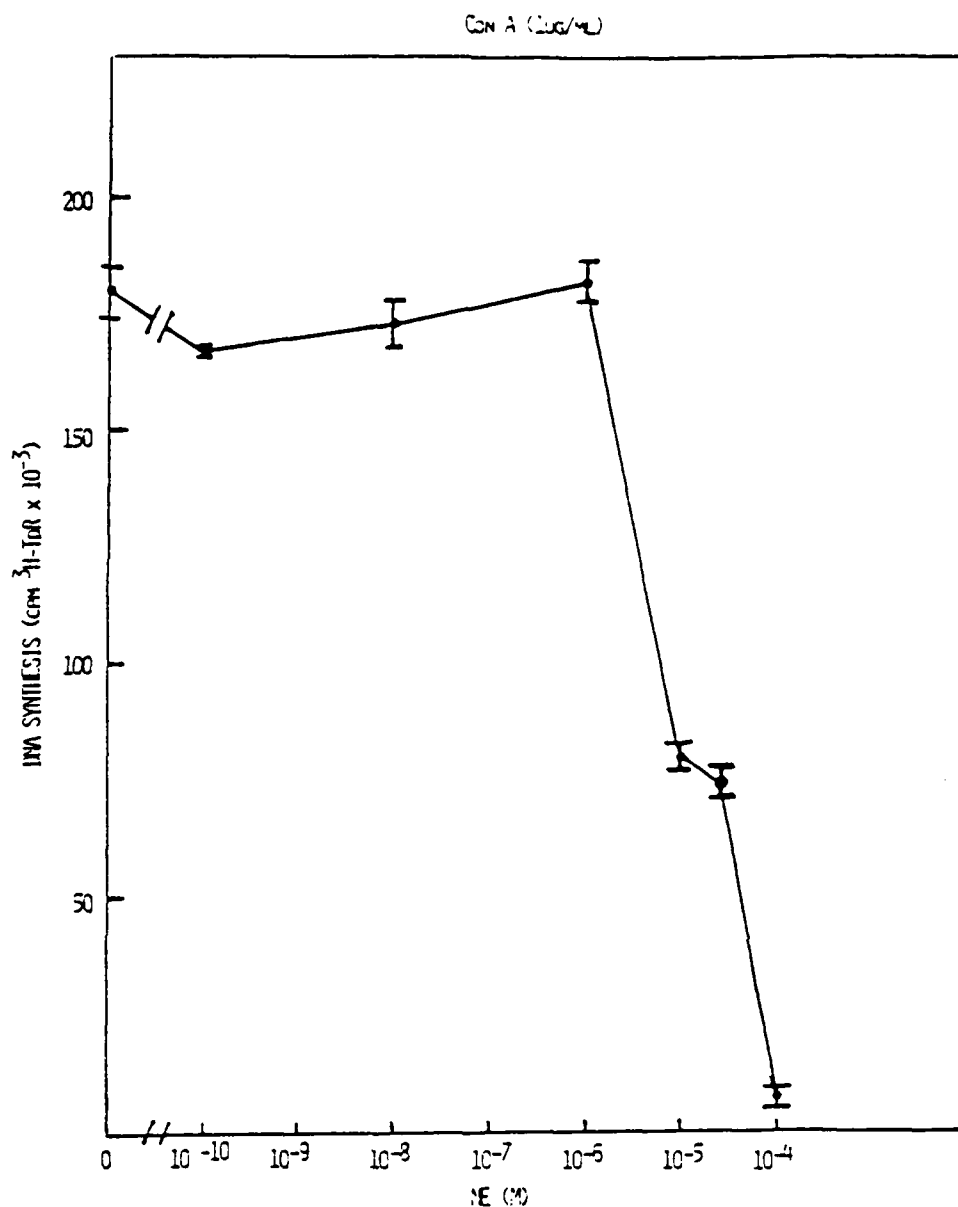


Figure 1. Effect of increasing NE concentration on BALB/c mouse spleen lymphocyte DNA synthesis. Addition of NE in ConA-stimulated DNA synthesis demonstrates maximal effect at 10^{-4}M .

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%.

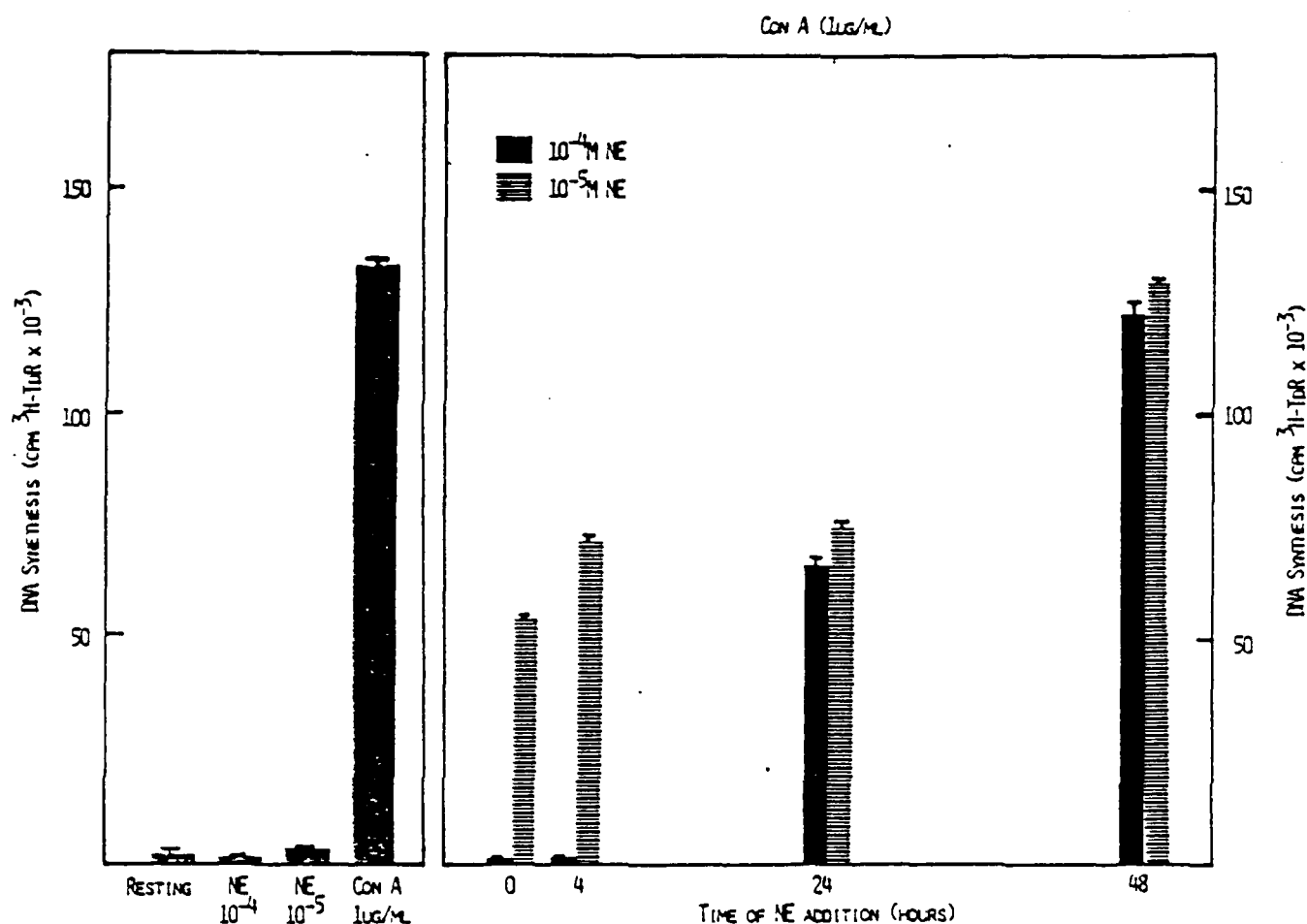


Figure 2. Effect of varying time of NE addition on DNA synthesis by ConA-stimulated BALB/c mouse spleen lymphocytes revealed maximal effect by 24 hours of culture.

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%.

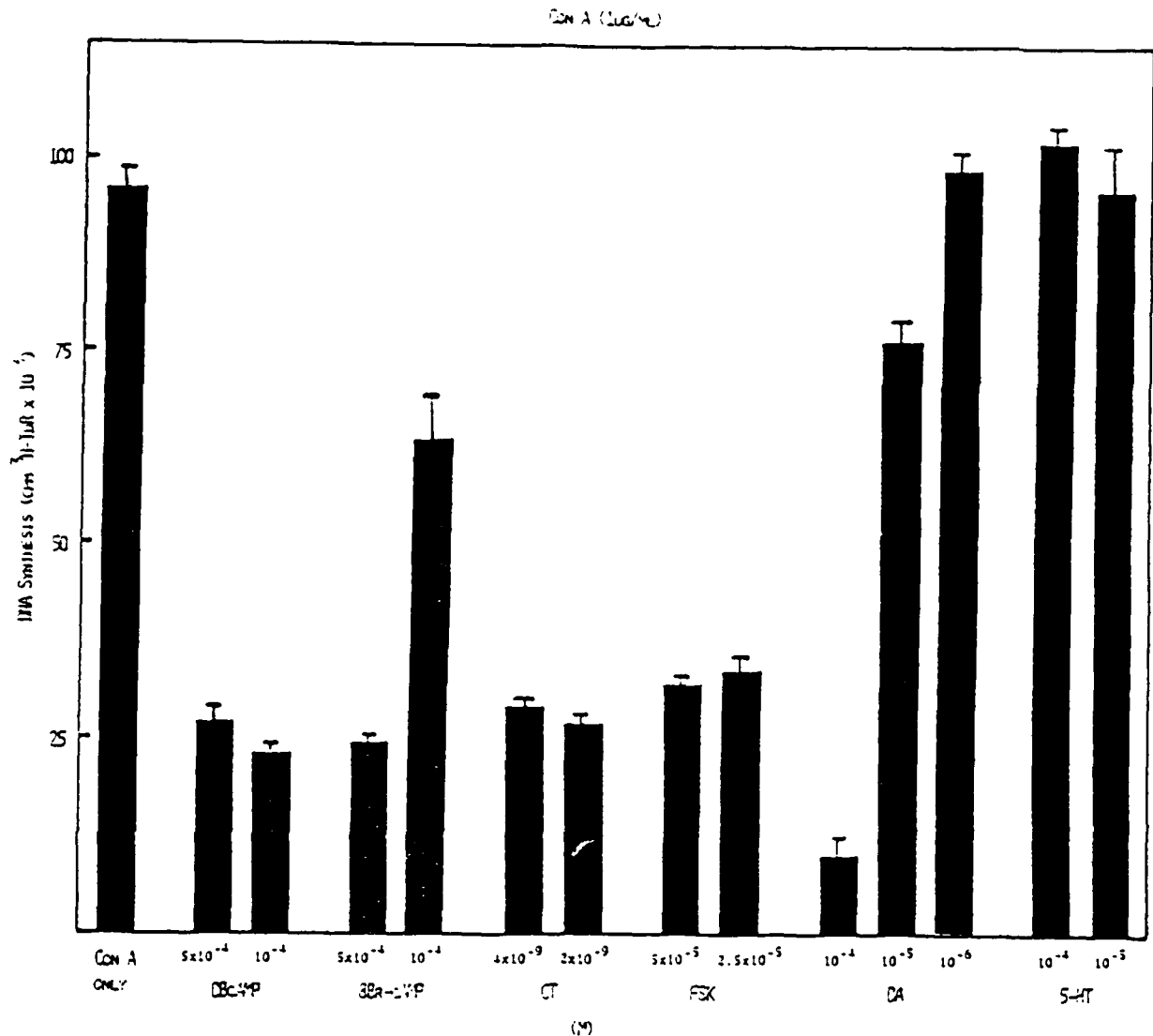


Figure 3. Cyclic AMP, cholera toxin and forskolin and dopamine can inhibit ConA-stimulated DNA synthesis by BALB/c spleen lymphocytes in contrast to 5-hydroxytryptamine.

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%.

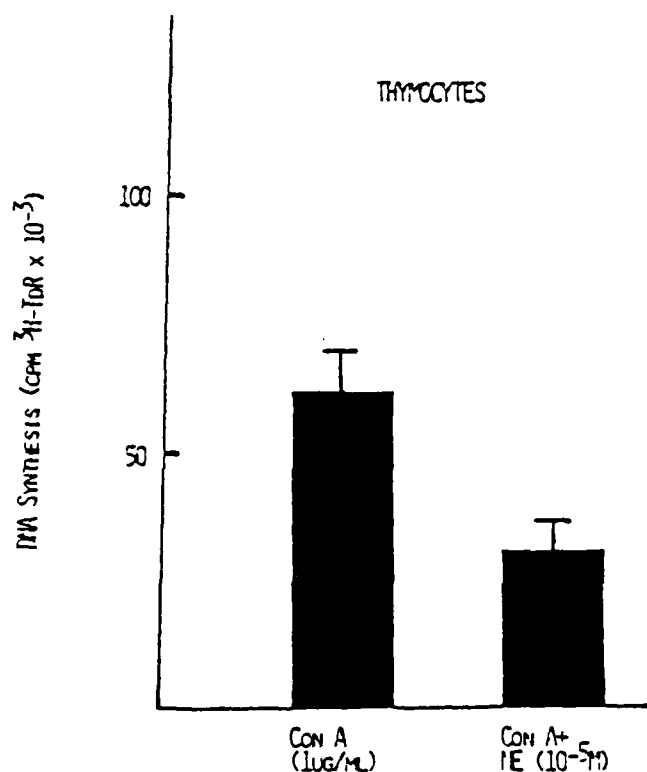


Figure 4. Addition of NE suppresses ConA-induced DNA synthesis in BALB/c mouse thymocytes.

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%.

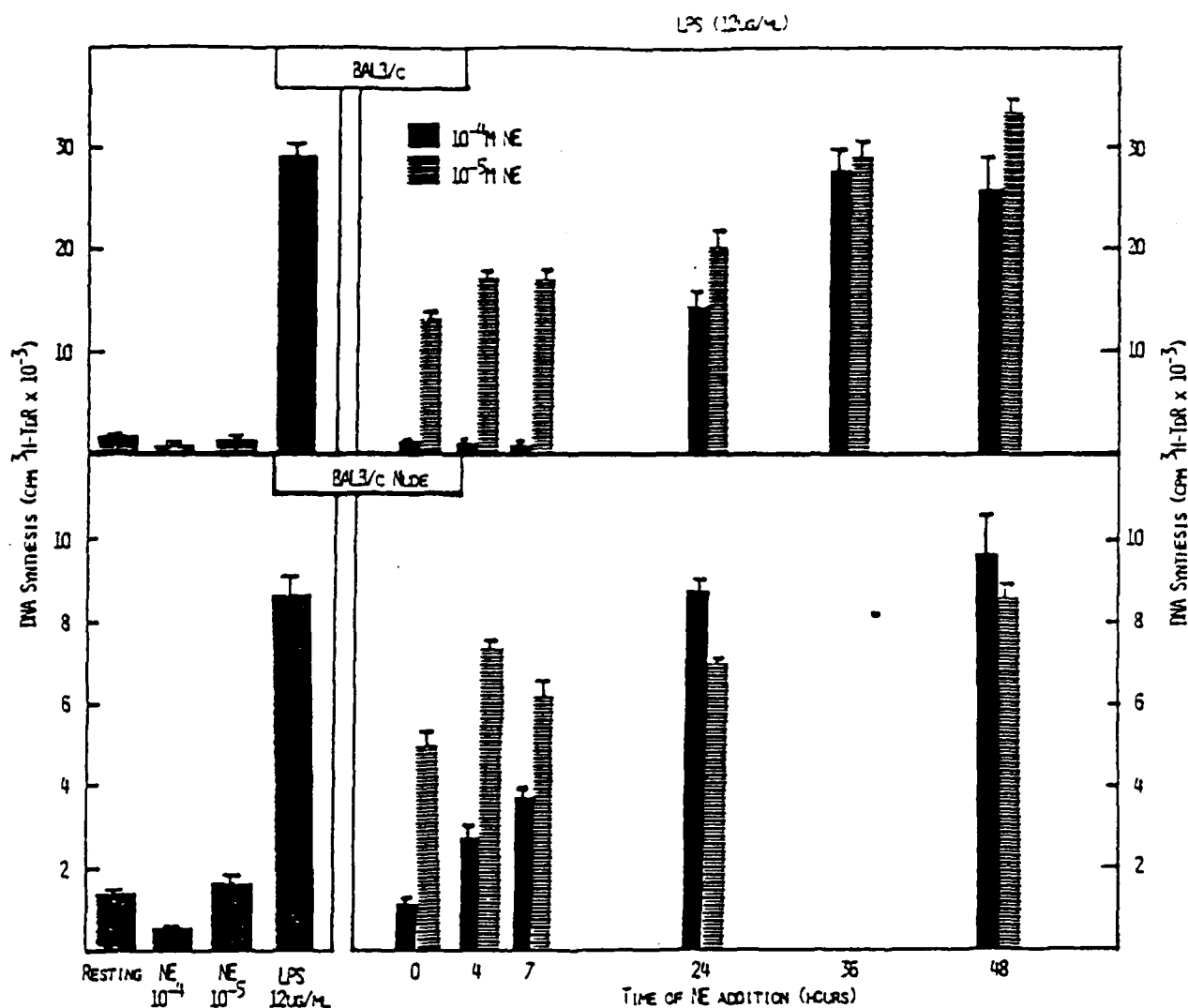


Figure 5. Addition of NE to normal or athymic (nude) mouse spleen cells at varying times after stimulation with the B-cell mitogen lipopolysaccharide reveals maximal suppression of DNA synthesis occurs within 24 hours of LPS addition.

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%.

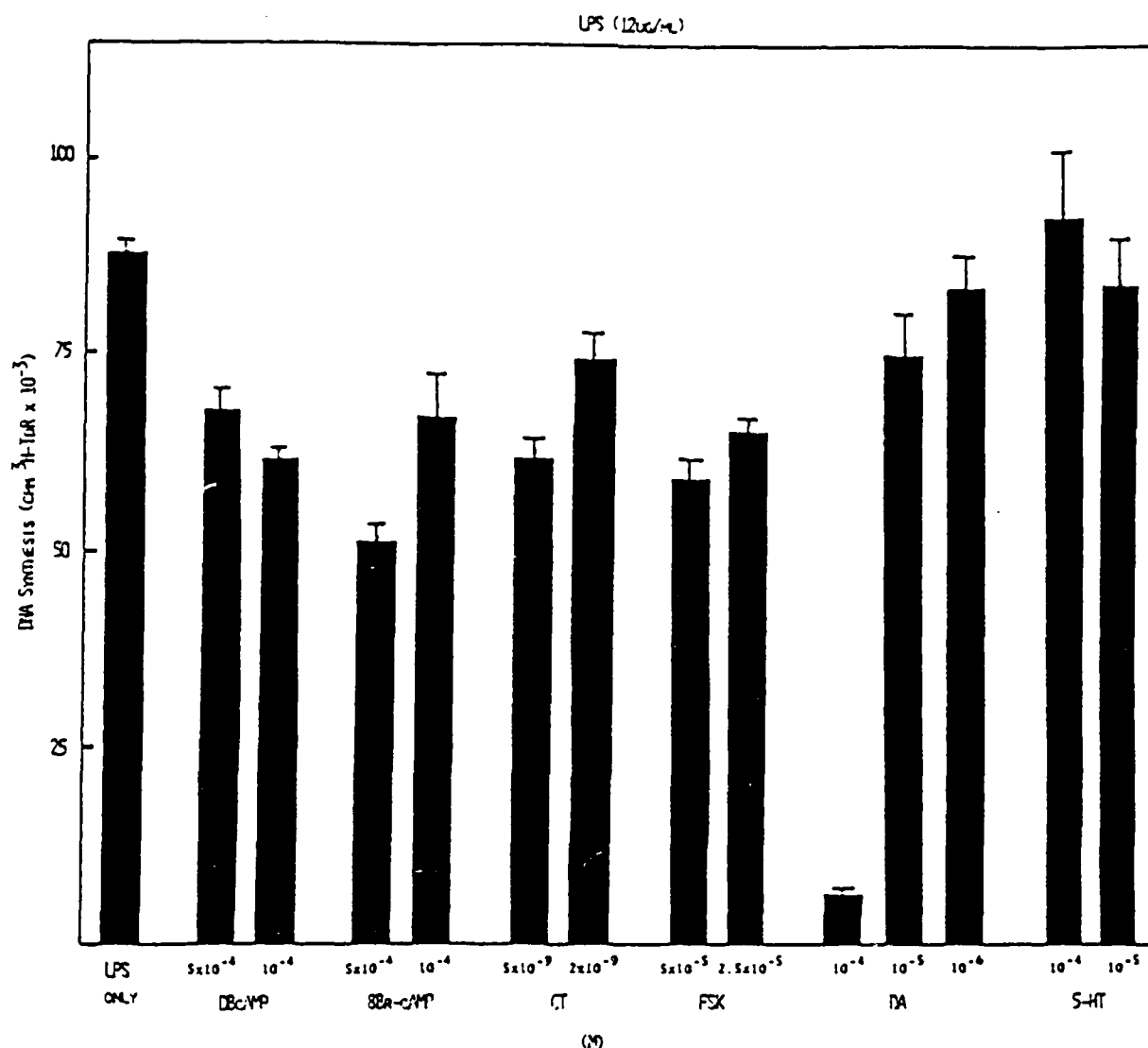


Figure 6. Cyclic AMP, cholera toxin, forskolin and 5-hydroxytryptamine had no effect on LPS-stimulated DNA synthesis by BALB/c spleen lymphocytes.

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%.

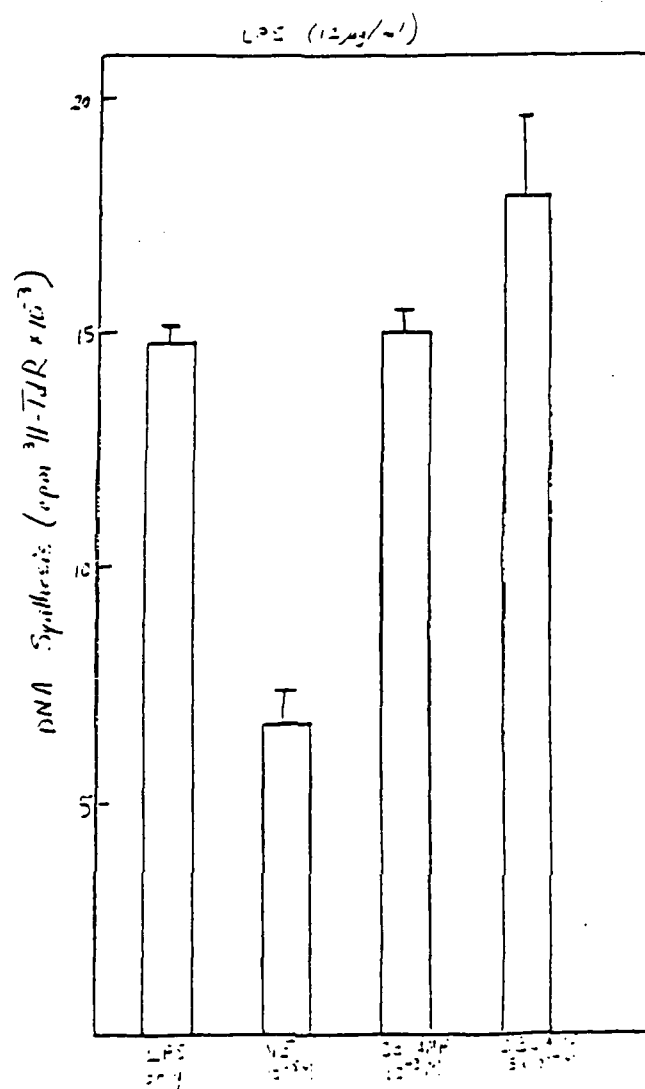


Figure 7. cAMP did not inhibit LPS-stimulated DNA synthesis by athymic (nude) mouse lymphocytes.

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 μ M) for 4 h. (3 H)DNA was measured by liquid scintillation counting. All experiments were run in quintuplicate. Variation was within 10%.

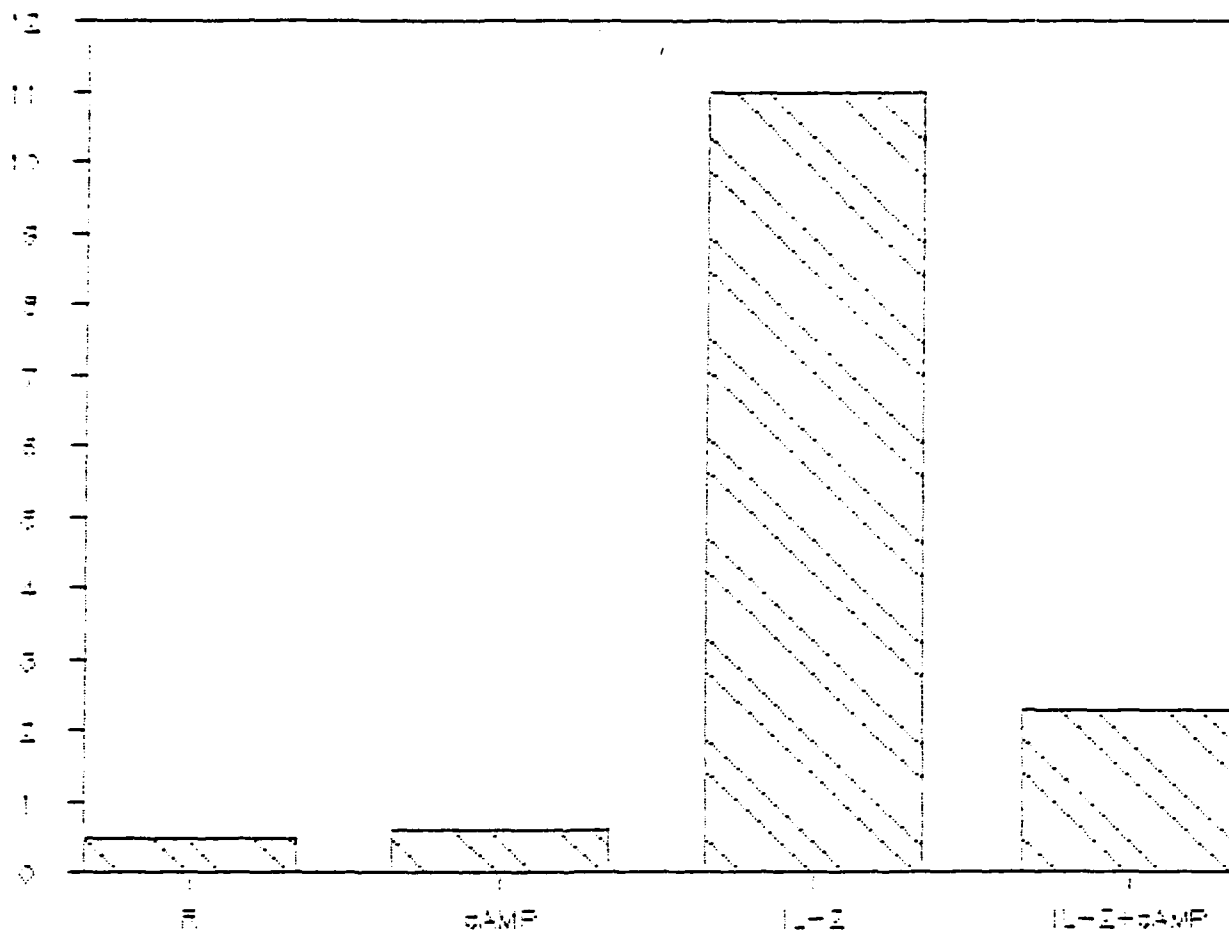


Figure 8. Cyclic AMP inhibited IL-2-stimulated spleen lymphocyte DNA synthesis.

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%.

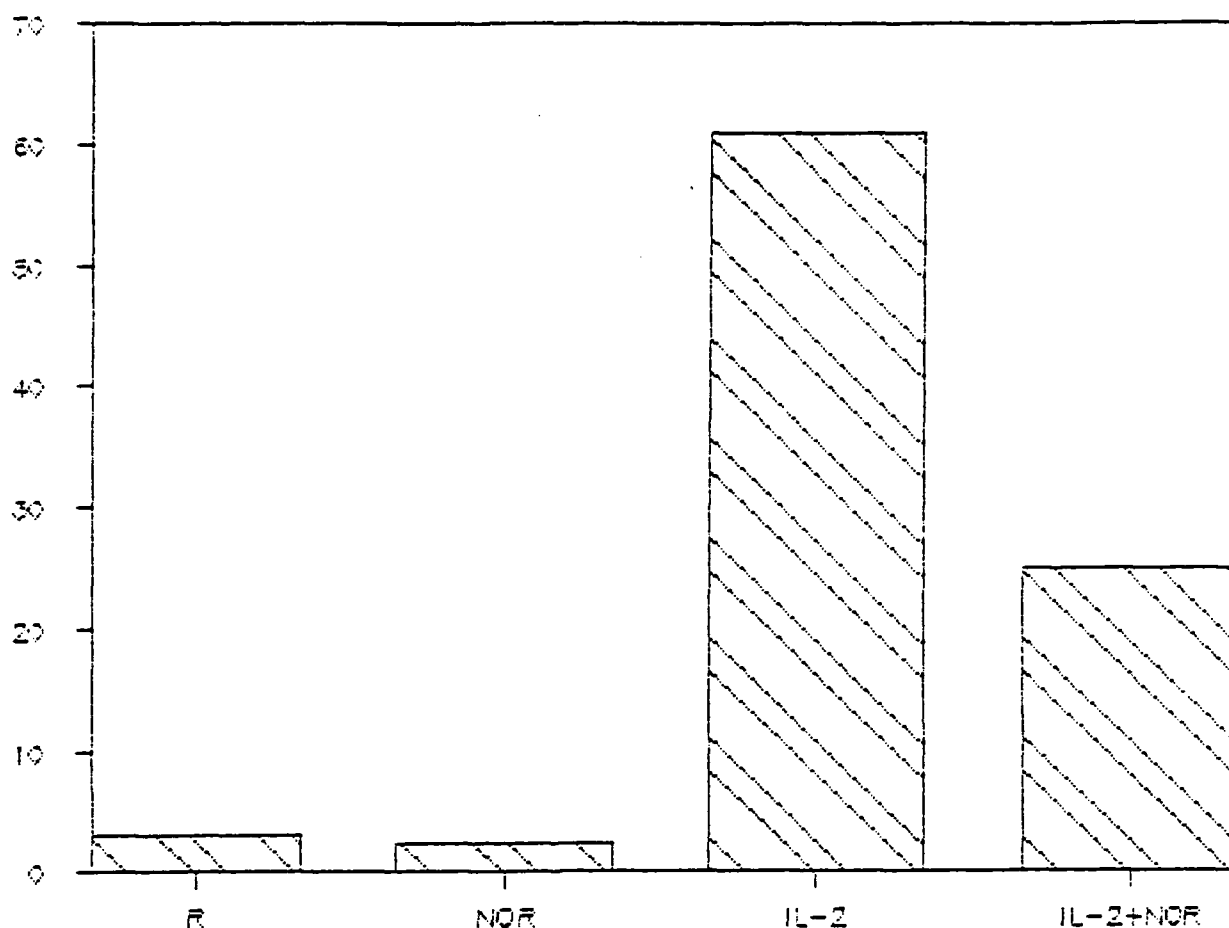


Figure 9. NE inhibited IL-2-stimulated spleen lymphocyte DNA synthesis.

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%.

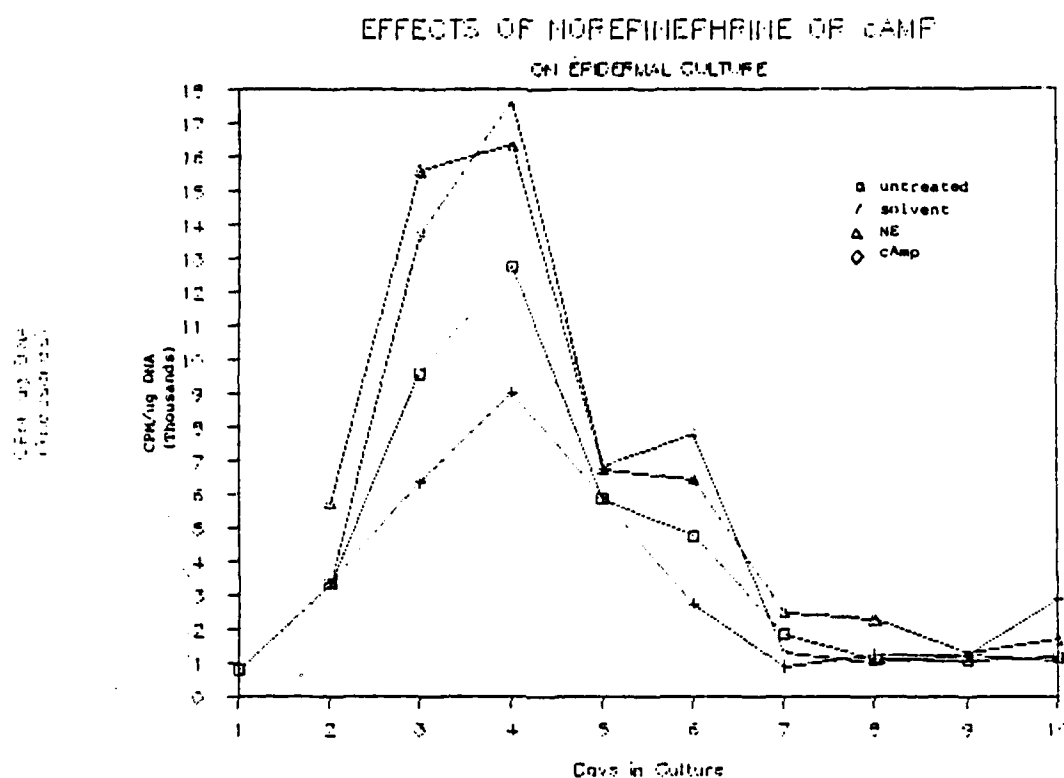


Figure 10. Addition of NE or cAMP lead to enhanced DNA synthesis by BALB/c mouse epidermal cells in culture.

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

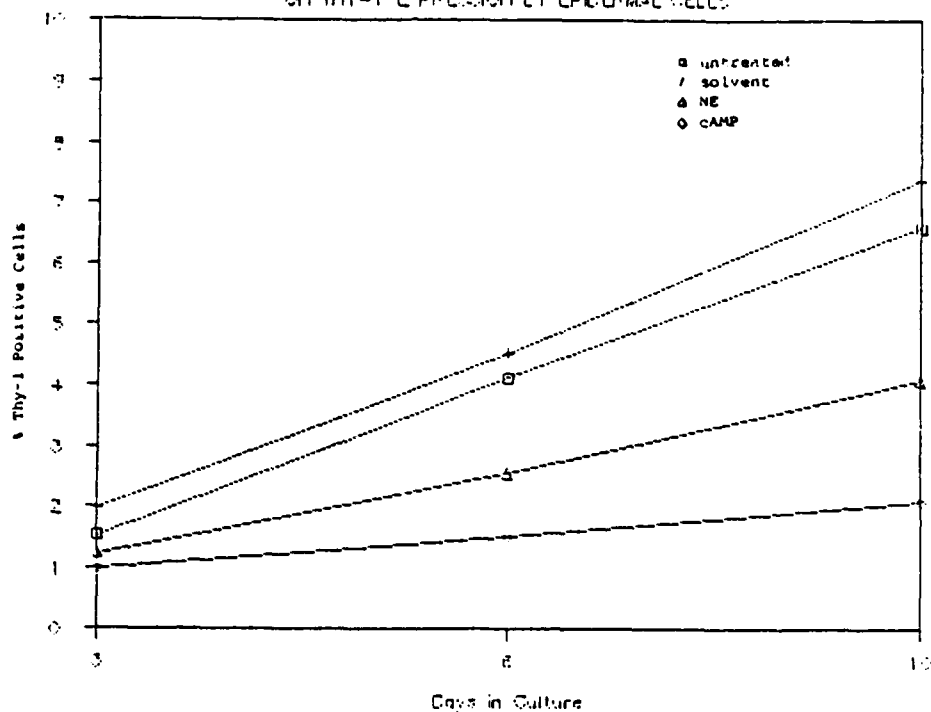


Figure 11. Thy-1 protein expression by neonatal BALB/c mouse epidermal cells in culture was diminished by addition of NE (10^{-6} M), cAMP (10^{-3} M) or cholera toxin (10^{-9} M). Epidermal cells were cultured as described in Figure 10, released into single cell suspension by brief incubation with EDTA 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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INVESTIGATORS

Dr. Itamar B. Abrass
Department of Medicine
University of Washington
Harborview Medical Center
Seattle, WA 98104

Dr. Prince K. Arora
NIDDK, Bldg. 8, Rm. 111
National Institutes of Health
Bethesda, MD 20892

Dr. Andrew S. Baum
Department of Medical Psychology
Uniformed Services University
of Health Sciences, B3050
4301 Jones Bridge Road
Bethesda, MD 20814-4799

Dr. Charles A. Bowles
Merrifield Research Lab, Inc.
P.O. Box 2362
Merrifield, VA 22116-2362

Dr. Karen Bulloch
Helicon Foundation
4622 Santa Fe Street
San Diego, CA 92109

Dr. Michael D. Cahalan
Department of Physiology and Biophysics
University of California, Irving
Irvine, CA 92717

Dr. Donald A. Chambers
Health Sciences Center
University of Illinois at Chicago
P.O. Box 6998
Chicago, IL 60680

Dr. Christopher L. Coe
Department of Psychology
Harlow Primate Laboratory
University of Wisconsin
Madison, IL 53715

Dr. Sheldon Cohen
Department of Psychology
Carnegie-Mellon University
Pittsburgh, PA 15213

Dr. Walla L. Dempsey
Dept. of Microbiology and
Immunology
The Medical College of
Pennsylvania
3300 Henry Avenue
Philadelphia, PA 19129

Dr. David L. Felten
Department of Anatomy
University of Rochester
School of Medicine
601 Elmwood Avenue
Rochester, NY 14642

Dr. John F. Hansbrough
Department of Surgery
UCSD Medical Center
225 Dickinson Street
San Diego, CA 92103

Dr. Robert L. Hunter
Department of Pathology
Emory Univ. School of
Medicine
WMB 760
Atlanta, GA 30322

Dr. Terry C. Johnson
Division of Biology
Ackert Hall
Kansas State University
Manhattan, KS 66506

Dr. Sandra Levy
University of Pittsburgh
School of Medicine
3811 O'Hara Street
Pittsburgh, PA 15213

Dr. Lester Luborsky
Department of Psychiatry
308 Piersol Building/GI
University of Pennsylvania Hospital
Philadelphia, PA 19104

Dr. Steven F. Maier
Department of Psychology
University of Colorado
Campus Box 345
Boulder, CO 80309

Dr. Diana S. Malcolm
Department of Surgery, USUHS
Uniformed Services University
of Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799

Dr. Michael H. Melner
Department of Reproductive Biology
Oregon Regional Primate Center
505 N.W. 185th Avenue
Beaverton, OR 97006

Dr. Vera B. Morhenn
Department of Dermatology
Stanford University Medical School
Stanford, CA 94305

Dr. Jose R. Perez-Polo
Gail Borden Bldg., Rm. 436
University of Texas Medical Branch
Galveston, TX 77550-2777

Dr. Howard R. Petty
Department of Biological Sciences
Wayne State University
Detroit, MI 48202

Dr. Bruce S. Rabin
Clinical Immunopathology
Childrens Hospital
University of Pittsburgh School of
Medicine
Pittsburgh, PA 15213

Dr. Seymour Reichlin
Director, Clinical Study Unit
New England Medical Center Hospitals, Inc.
171 Harrison Avenue
Boston, MA 02111

Dr. Eric M. Smith
Department of Psychiatry
University of Texas
Medical Branch
Galveston, TX 77550

Dr. Ross R. Vickers, Jr.
Naval Health Research Ctr.
Bldg. 346
P.O. Box 85122
San Diego, CA 92138

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ADMINISTRATORS

Dr. Jeannine A. Majde, Code 1141SB (2 copies)
Scientific Officer, Immunology Program
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Arlington, VA 22217-5000

Program Manager
Biological/Human Factors Division
Office of Naval Research, Code 12
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Arlington, VA 22217-5000

Administrator (2 copies) (Enclose DTIC Form 50)
Defense Technical Information Center
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Alexandria, VA 22314

Program Manager
Support Technology Directorate
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