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13. ABSTRACT (Maximum 200 Words) L-deprenyl, a monoamine oxidase-B inhibitor, has been reported to reverse the age-related decline in sympathetic noradrenergic innervation and immune function in old rats and enhance cell-mediated immunity in tumor-bearing rats. The aim of the present set of studies was to investigate whether deprenyl treatment of young and old female rats with carcinogen-induced and spontaneously developing mammary tumors, and middle-aged female rats could inhibit tumor development and growth. Also, the effects of deprenyl treatment on splenic noradrenergic activity and immune responses and hypothalamic dopaminergic activity were examined. In another study, we investigated whether deprenyl has any direct effects on the growth of breast cancer cell lines. We have demonstrated that deprenyl inhibited the development and growth of tumors in young and old rats with tumors and suppressed the incidence of tumors when deprenyl treatment is initiated in middle-aged female rats. These effects were accompanied by increase in immune reactivity and sympathetic noradrenergic activity in the spleen and hypothalamic dopaminergic activity. Direct addition of deprenyl to breast cancer cell lines moderately inhibited the growth of cells. These results suggest that the anti-tumor effects of deprenyl involve neural-immune signaling that may be responsible for anti-mitogenic effect at the cellular level.				
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Introduction

Advances in breast cancer research have changed the prognosis for the cancer from an incurable to a manageable level in many women through a variety of treatment procedures. Although a combination of chemotherapy and surgery has increased the survivability and reduced the mortality, further research is needed, as indicated by the fact that there is still an increase in the incidence of breast cancer (1, 2). The pathogenesis of breast cancer is complex and is not attributable to a single factor but to an array of inter-linked factors such as hormonal status, age at first live birth, genetic predisposition, diet, environmental factors, etc. These factors influence the carcinogenesis through a combination of physical, biochemical, and genetic, damage to the involved cells in all the three steps including initiation, promotion, and progression of tumor growth. Although the etiology of breast cancer is not clear, it is generally believed that the environmental factors contribute in a major way in initiating the carcinogenesis that involves changes at the genetic level (3, 4). We believe that these effects at the cellular level are mediated through alterations in a number of systems including the nervous, immune, and the endocrine systems.

Body

Statement of Work: Task 6: Months 13-16: Analyses of samples and data collected in expt. 1A.

Treatment of rats with dimethylbenzanthracene (DMBA) -induced mammary tumors with deprenyl inhibited development and growth of mammary tumors, increased natural killer (NK) cell activity, interferon- γ (IFN- γ) and interleukin-2 (IL-2) production, central and peripheral catecholaminergic activity. An interesting finding was that saline-treated rats with progressively growing tumors had suppressed NK cell activity, IFN- γ and IL-2 production accompanied by decreased central and peripheral catecholaminergic activity. The details of the experiments and results have been published in Journal of Neuroimmunology 109: 95-104, 2000 (please refer to the Appendix).

Task 12: Months 33-36: Analyses of samples and data from expt. 2.

When rats with spontaneously occurring mammary tumors were treated with deprenyl, there was a decrease in tumor growth and enhanced IFN- γ production, percentage of CD8+ T cells in spleen, and Con A-induced proliferation of lymphocytes accompanied by increased catecholaminergic activity in the medial basal hypothalamus (MBH) and spleen. Please refer to the Appendix for the details on the procedures, results and discussion (Anticancer Res., 19:5023-5028, 1999).

Task 3: Months 30-31: Analysis of samples and data from expt. 1B (UNPUBLISHED DATA).

We have preliminary evidence that deprenyl has direct inhibitory effect on the cells of breast cancer cell lines. This anti-proliferative effect is more pronounced in estrogen receptor-positive breast cancer cell lines than in estrogen receptor-negative cell lines. Further experiments are essential to understand the effects of deprenyl on cell cycle and the cell cycle regulatory proteins and gene products.

Materials and Methods: Cells were plated at a concentration of 5,000 cells/well. On Day 0, freshly prepared media containing L-deprenyl (10^{-3} to 10^{-8} M) was added to the cells. Thereafter, freshly prepared media with the drug was changed every other day for a total of 6 days. On the days for reading viable cells, the one-solution MTS tetrazolium compound reagent

([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS and phenazine ethosulfate; PES) was added to each well and was incubated for 1-4 hours. The absorbance was read at 490nm with a microplate reader. The MTS tetrazolium compound (Owen's reagent) is bioreduced by live cells into a colored formazan product that is soluble in culture medium and is directly proportional to the number of live cells in culture. A standard concentration curve of known cell concentration versus absorbance was plotted and used to determine the number of viable cells from the recorded absorbance.

Results: The estrogen receptor positive breast cancer cell lines (MCF-7 and T47-D) show significant reduction in the number of cells following incubation with deprenyl. The effects of addition of deprenyl to MCF-7 cells in inhibiting cell growth were more pronounced on day 1 and day 6 (Fig. 1; $P < 0.05$). Similarly, deprenyl significantly inhibited the growth of cells in T47-D cell line on all the days (Fig. 2). The lower doses of deprenyl were effective in suppressing the growth of cells on days 4 and 6.

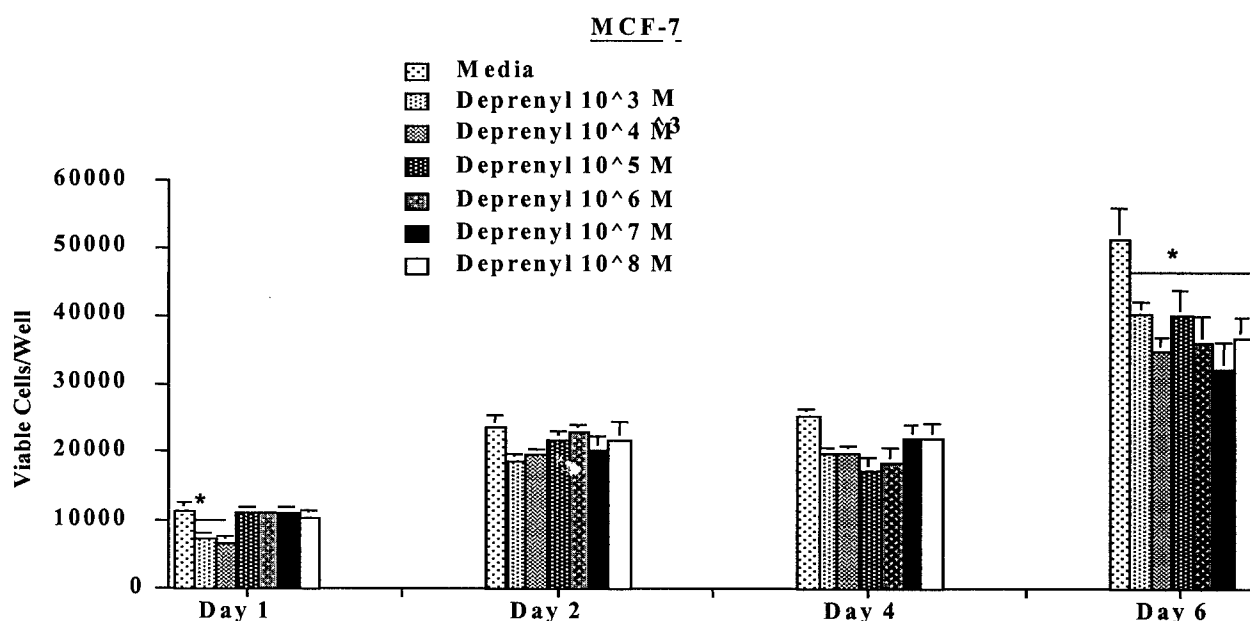


Fig. 1: Estrogen receptor-positive breast cancer cell line, MCF-7, was plated at a initial concentration of 5000 cells/well on day 0 and media/deprenyl was added on alternated days to determine cell viability. Addition of deprenyl significantly inhibited the growth of cells on day 6.

The estrogen receptor negative breast cancer cell lines, MDA-MB-231 and Hs578-T, were unresponsive to the effects of deprenyl except on day 4 in Hs578-T breast cancer cell line (Figs. 3 and 4). Further studies are being done to determine whether the inhibitory effect of deprenyl is very specific to this cell line.

These results demonstrate that deprenyl's effects on breast cancer cell lines are dependent upon the estrogen receptor status. It would be ideal to confirm these results by examining the effects of deprenyl on (1) the stages of cell cycle, (2) estrogen receptor binding, and (3) cell cycle proteins and gene products. Some of the studies have shown that deprenyl is capable of modulating the genes responsible for apoptosis, bcl-2 and bax. We are proposing to test whether similar effects of deprenyl on the apoptotic genes can be observed on breast cancer cell lines.

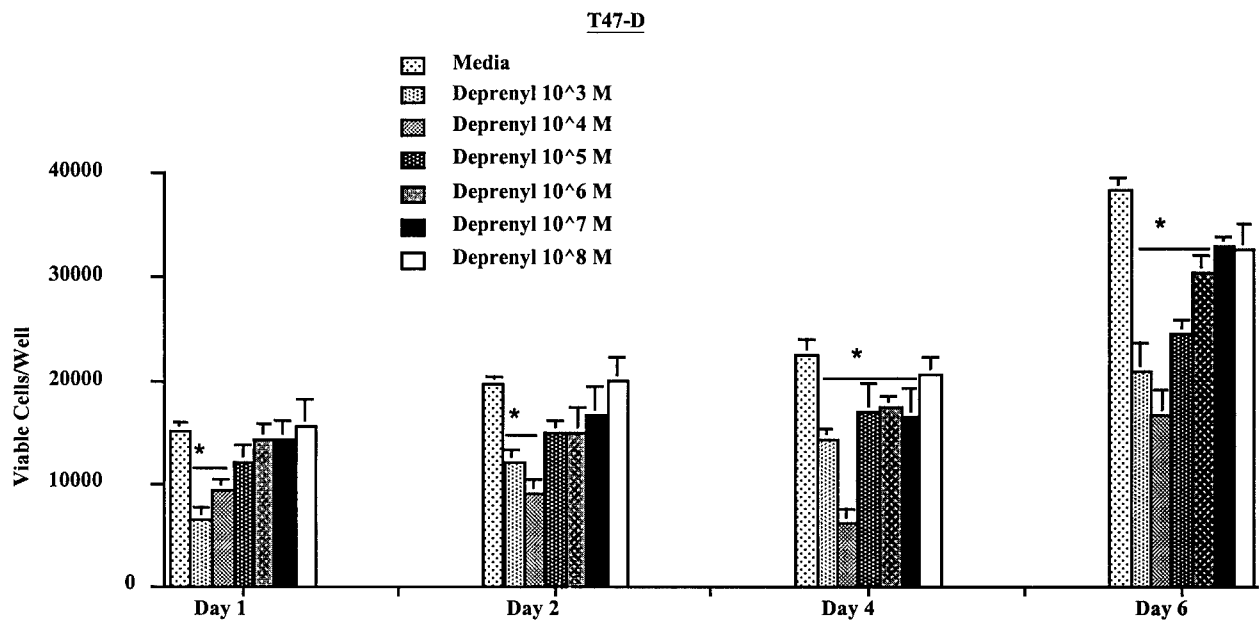


Fig. 2: Estrogen receptor-positive breast cancer cell line, T47-D, was plated at a initial concentration of 5000 cells/well on day 0 and media/deprenyl was added on alternated days to determine cell viability. Addition of deprenyl significantly inhibited the growth of cells on days 1-6 with lower doses of deprenyl being effective on days 4 and 6.

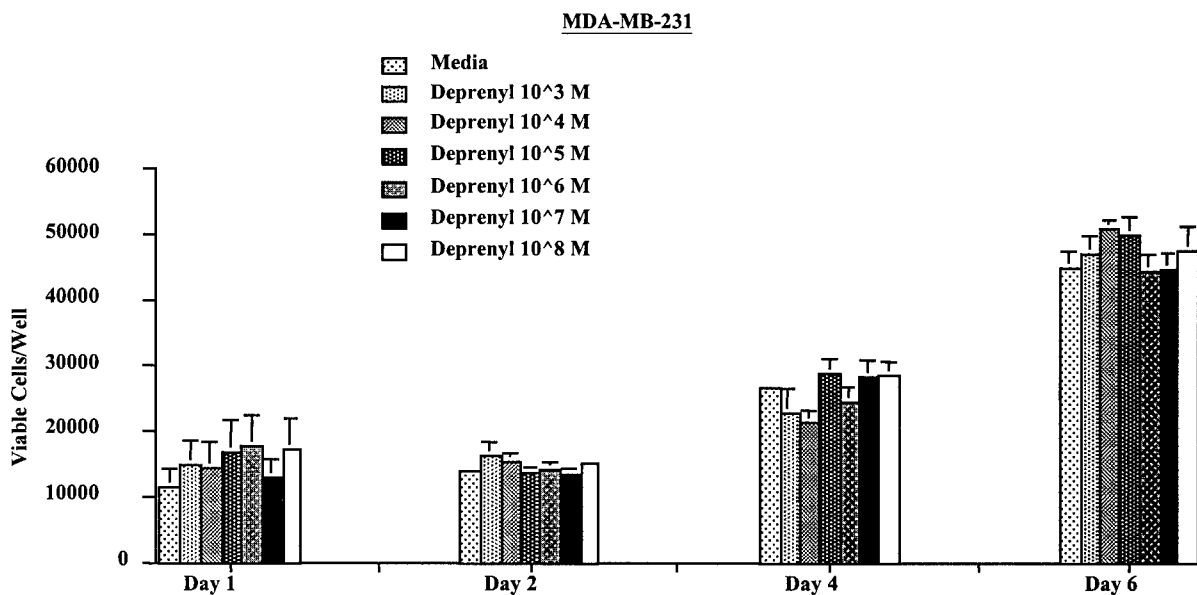


Fig. 3: Estrogen receptor-negative breast cancer cell line, MDA-MB-231, was plated at a initial concentration of 5000 cells/well on day 0 and media/deprenyl was added on alternated days to determine cell viability. Addition of deprenyl did not alter the growth of breast cancer cells.

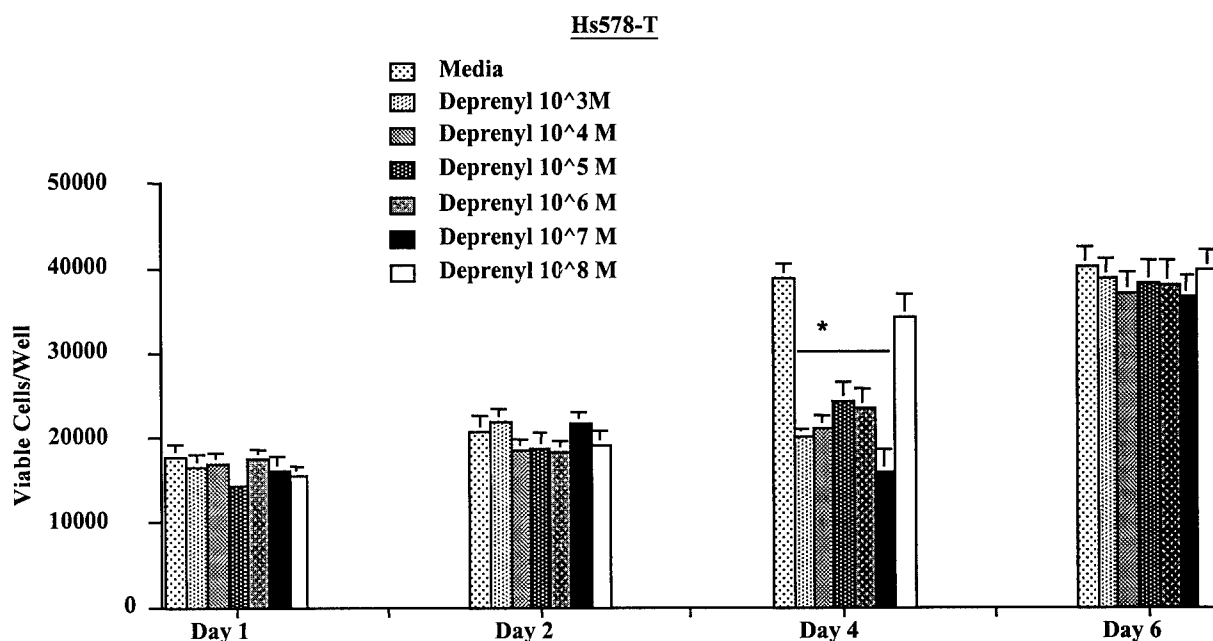


Fig. 4: Estrogen receptor-negative breast cancer cell line, Hs578-T, was plated at a initial concentration of 5000 cells/well on day 0 and media/deprenyl was added on alternated days to determine cell viability. Addition of deprenyl significantly inhibited the growth of cells on day 4.

Discussion: In this study, we have demonstrated that deprenyl is capable of inhibiting the growth of breast cancer cells. This direct effect of deprenyl on breast cancer cells may be one of the mechanism by which deprenyl inhibits the growth of mammary tumors in young and old female rats with tumors (5, 6). Further studies are essential to understand the specific inhibitory effects of deprenyl on estrogen receptor (ER)-positive breast cancer cell lines. This effect may be dependent upon the estrogen receptor binding or activation of estrogen receptor. Deprenyl may interfere with alteration in estrogen receptor translocation to the nucleus, inhibition of genetic expression of cytoplasmic estrogen receptor, and inhibition of the activity of calmodulin and/or protein kinase C to modulate the activation of estrogen receptor in ER positive breast cancer cell lines (7-10). Another possibility is ER-dependent growth factor or growth factor receptor activity in breast cancer cells may play a critical role in deprenyl-induced suppression of growth of cells (11-13).

A number of genes and their protein products have been known to influence the apoptotic process. Of these genes, deprenyl has been shown to influence the expression of bcl-2 and bax, some of the genes that regulate apoptosis in neuronal cell cultures (14). It is possible that deprenyl influences the expression of these genes through modulation of phosphorylation of c-JUN NH₂-terminal kinase (JNK) and JNK kinase (15).

Our results demonstrate that the anti-tumor effects of deprenyl is dependent upon both direct inhibitory effect on the cellular growth and indirectly modulating the neuroendocrine and immune systems. These effects may be regulated by another important property of deprenyl to enhance the activities of antioxidant enzymes. Free radicals have been shown to alter the immune responses and also promote the growth of cancer cells and deprenyl is known to promote the activities of superoxide dismutase, catalase and glutathione peroxidase (16-19).

Task 5: Months 34-36: Completion of expt. 3. Analysis of samples and data from expt. 3 (UNPUBLISHED DATA).

In a preliminary study in our laboratory, we found that deprenyl is capable of protecting the tuberoinfundibular dopaminergic system in the medial basal hypothalamus and inhibiting the development and the incidence of tumors in rats with carcinogen-induced mammary tumors (20). The development of tumors is not a sudden event but a culmination of prolonged alterations in the neuroendocrine-immune communication network. The neuroendocrine system is altered in the middle-aged rats, characterized by changes in the estrous cyclicity, hypothalamic catecholamines, and level of gonadal hormones (21-24).

Materials and Methods: The aim of this study is to determine whether initiating deprenyl treatment in middle-aged female rats (8-9 mo; n=30/group) would prevent the development and growth of mammary tumors at a later age. Saline and two doses of deprenyl (1.0 and 2.5 mg deprenyl/kg body weight) were injected intraperitoneally every day and tumor development was monitored every week for a period of 12 months. A group of young female rats (3 mo; n=10) was sacrificed at the end of the treatment period to compare the age-related effects on the various parameters to be measured in this study. At the end of the study, spleens were dissected out and used for NK cell activity, Con A-induced proliferation of lymphocytes, and flow cytometry. Lymph nodes (inguinal and axillary) were isolated and pooled for measuring Con A-induced proliferation of lymphocytes.

NK cell activity: NK cell activity was assessed using the NK-sensitive lymphoma YAC-1 passaged in vitro. YAC-1 cells in log phase growth were incubated with 100 μ Ci of $\text{Na}_2^{51}\text{CrO}_4$ (DuPont NEN, Boston, MA) at 37°C for 90 min. The cells were washed three times and adjusted to 10^5 cells/ml. Spleen cells ratios were mixed with 10^4 ^{51}Cr -labeled YAC-1 cells at varying effector to target (E:T) ratios in round-bottom 96-well tissue culture plates (Falcon, Becton Dickinson) in triplicate in a volume of 200 μ l. Spontaneous release was determined by incubating 10^4 ^{51}Cr -labeled YAC-1 cells with complete RPMI alone. Maximum release was determined by adding 1% Triton X-100 to 10^4 ^{51}Cr -labeled YAC-1 cells. The plates were then centrifuged at 200 g for 5 min and incubated for 4 h at 37°C in a CO_2 -humidified atmosphere. The plates were centrifuged at 500 g for 5 min at 4°C, and 100 μ l of supernatant was removed from each well, and radioactivity was counted in a gamma counter. Cytotoxic activity was expressed as percent lysis, determined by the equation (experimental cpm-spontaneous cpm)/(maximum cpm-spontaneous cpm) X 100.

Con A-induced proliferation

Spleen cells and lymph node cells, 2×10^5 cells/well, were cultured in triplicate with either medium alone or varying concentrations of Con A (Calbiochem-Behring Corp., La Jolla, CA), in 96-well, flat bottom tissue culture plates (Falcon), and maintained for 3 days at 37°C in 5% CO_2 -humidified incubator. [^3H]-Thymidine (0.5 μ Ci/10 μ l; 5 Ci/mmol; DuPont NEN, Boston, MA) was added for the final 18 h of culture. Cells were harvested on to glass fiber filter paper (Whatman Inc., Clifton, NJ) with a cell harvester (Skatron). The dried filters were placed in scintillation fluid (Biosafe II, RPI, Mount Prospect, IL), and radioactivity determined with a liquid scintillation counter (LKB, Wallac, Finland).

Flow cytometric analysis

Spleen cells were washed in PBS containing 2% BSA and 0.02% azide (flow wash). Fluorescein-conjugated anti-rat sIgM (clone G53-238, diluted 1:40; Pharmingen) and phycoerythrin-conjugated anti-NKR-P1A (an NK cell marker, clone 10/78, diluted 1:40; Pharmingen) or fluorescein-conjugated anti-rat CD8 (clone OX-8, diluted 1:40; Pharmingen) and phycoerythrin-conjugated anti-CD4 (clone OX-35, diluted 1:20; Pharmingen) were added to 2×10^6 cells and incubated at 4°C for 30 min. Cells incubated with flow wash alone were included to determine autofluorescence. Following this incubation, cells were washed twice in flow wash, fixed in PBS containing 1% paraformaldehyde, and stored in the dark for no longer than 2 weeks at 4°C prior to analysis. Two-color fluorescence was analyzed with an Elite flow cytometer (Coulter Electronics, Hialeah, FL), equipped with an argon-laser at 15 mW and excitation wavelength of 488 nm.

Statistical analysis

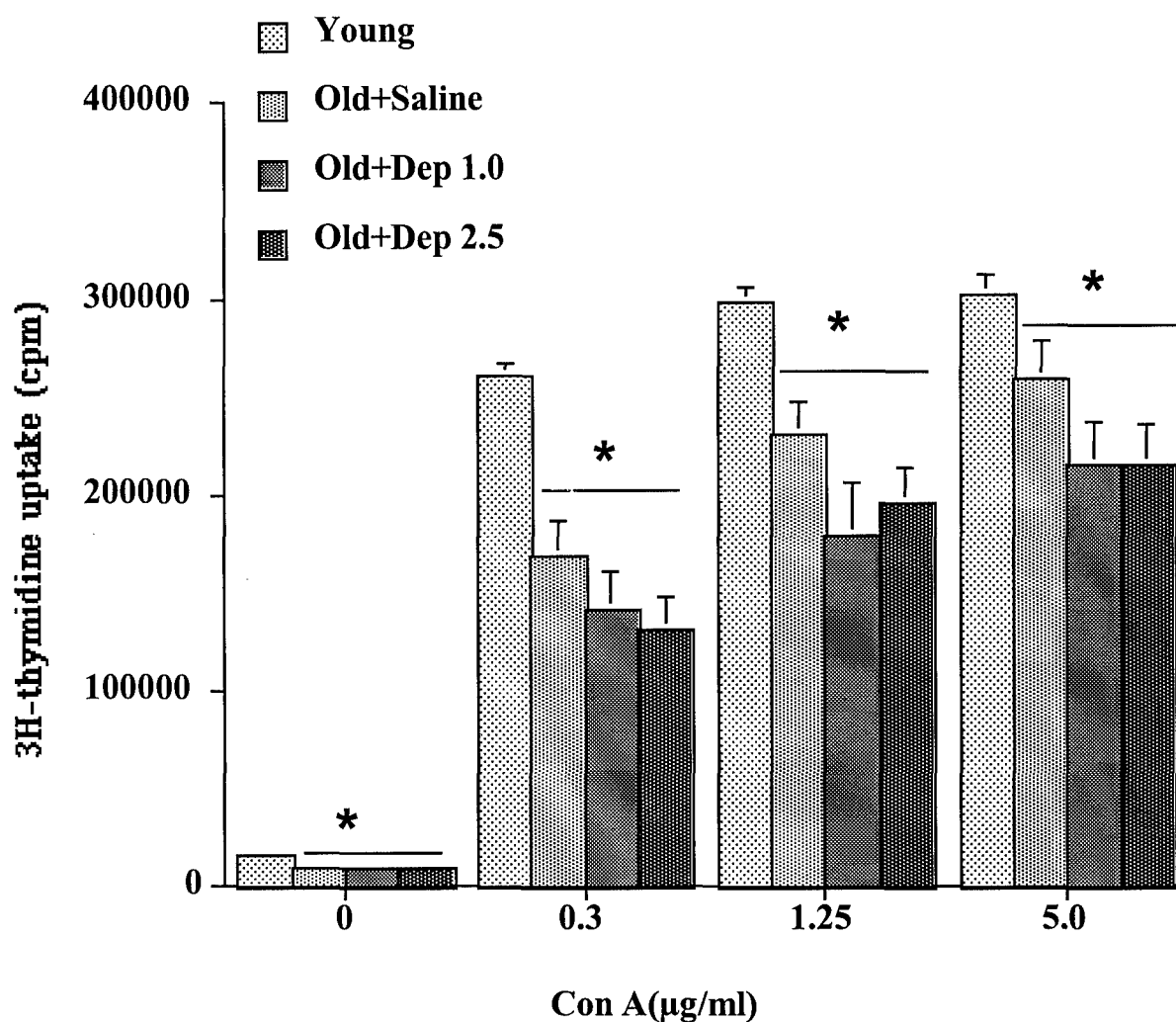
The data were analyzed by ANOVA. NK cell activity and Con A-induced proliferation were analyzed using ANOVA with E:T ratio and Con A concentration as repeated measures. Parameters that attained significance following ANOVA ($P < 0.05$) were further analyzed by Fisher's least significant difference test.

Results: The percentage of rats that developed tumor in the saline-treated group was 73% whereas the percentage of rats that developed tumor was significantly ($P < 0.05$) lower in rats that were treated with 1.0 mg/kg deprenyl (46%) and 2.5 mg/kg deprenyl (36%). Following the development of tumors, some of the tumors continue to grow in size irrespective of the treatment regimen. But some of the tumors regressed in deprenyl-treated groups.

Con A-induced T cell proliferation in the spleen was decreased in saline- and deprenyl-treated tumor-bearing rats in comparison to young animals (Figure 5). In contrast, Con A-induced proliferation of lymphocytes in the lymph nodes was higher in rats that were treated with 2.5 mg/kg deprenyl (Figure 6). Flow cytometric analysis of splenic B cell populations revealed a significant reduction in sIgM+ B cells in tumor-bearing rats (Figure 7). Deprenyl induced no alterations in the percentage of CD8+ T cells but there was a modest increase in CD4+ T cells in rats that received 2.5 mg/kg deprenyl.

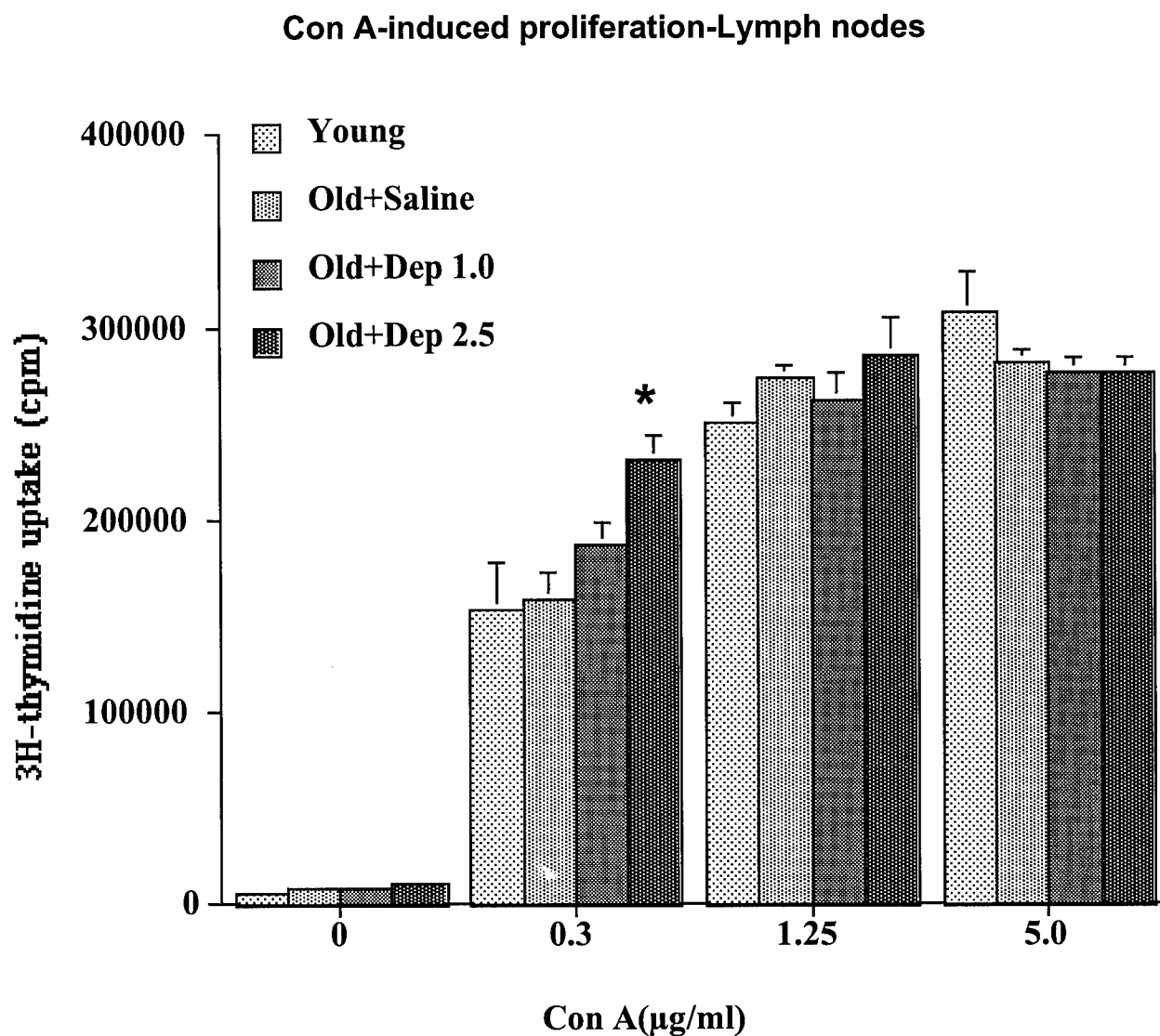
NK cell activity analyzed by ANOVA revealed a three-way interaction between treatment period, drug treatment and E:T ratio (Figure 8). NK cell activity at a higher ratio (190:1) was lower in tumor-bearing rats treated with saline compared to young animals without tumors, but it was higher in rats treated with 1.0 mg and 2.5 mg/kg deprenyl in comparison to saline-treated rats. NK cell activity was significantly increased at lower E:T ratios (90:1) in animals that received daily treatment of 2.5 mg/kg deprenyl compared to saline-treated groups. There was no significant increase in the percentage of NK cells associated with the increase in NK cell activity (Figure 7).

Con A-induced proliferation-Spleen



***Significantly different from Young**

Figure 5: Con A-induced T lymphocyte proliferation by spleen cells from rats with mammary tumors after 12 months of treatment with 0, 1.0 mg, or 2.5 m /kg BW/day of deprenyl. Spleen cells were incubated with 0, 0.3, 1.25, or 5 μg/ml of Con A for 72 hrs. Proliferation of T lymphocytes was reduced in saline- and deprenyl-treated rats in comparison to young rats.



***Significantly different from the other groups**

Figure 6: Con A-induced T lymphocyte proliferation by lymph node cells from rats with mammary tumors after 12 months of treatment with 0, 1.0 mg, or 2.5 mg/kg BW/day of deprenyl. Lymph node cells were incubated with 0, 0.3, 1.25, or 5 µg/ml of Con A for 72 hrs. Proliferation of T lymphocytes was increased in 2.5 mg deprenyl-treated rats in comparison to rats from other groups.

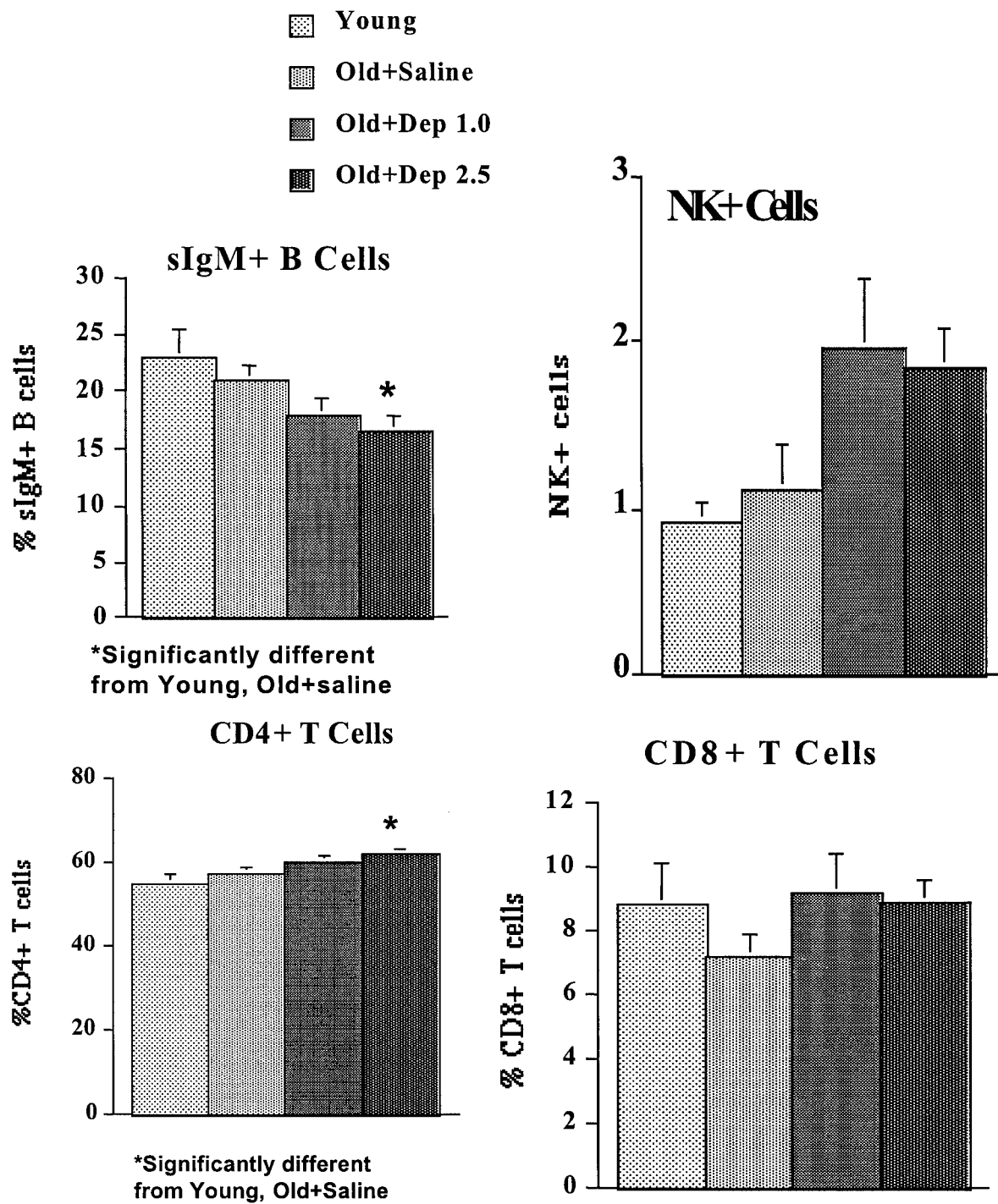


Figure 7: Spleen lymphocyte populations from rats with mammary tumors that were treated with saline and deprenyl for 12 months.

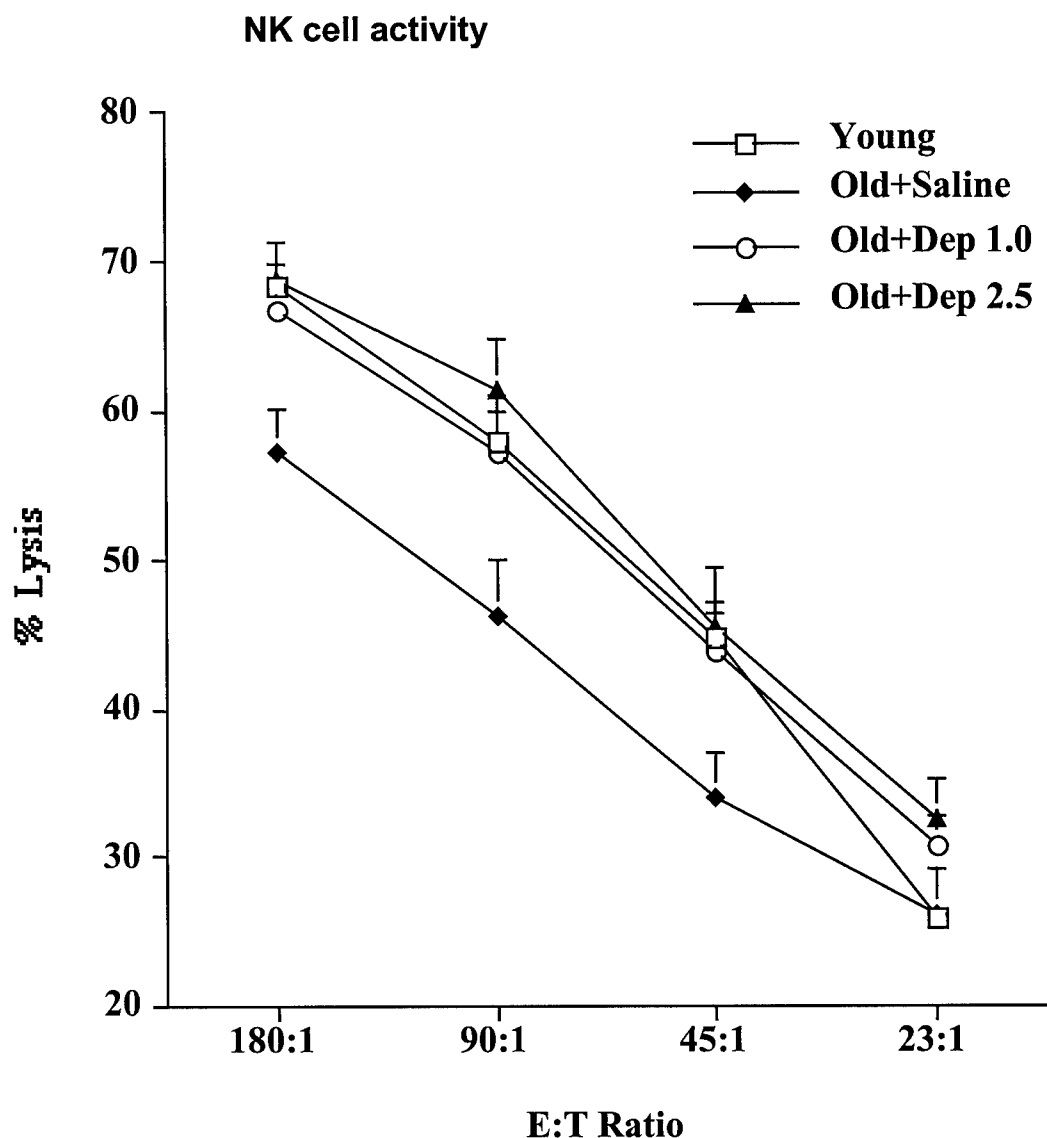


Figure 8: Splenic NK cell activity from rats with mammary tumors after 12 months of treatment with 0, 1.0 mg, or 2.5 m /kg BW/day of deprenyl.

Discussion: In this experiment, we have demonstrated that deprenyl treatment of middle-aged female rats partially prevents the development of mammary tumors with advancing age. The effect on tumor development was associated with increase in NK cell activity and Con A-induced proliferation of lymphocytes in lymph nodes.

There are profound changes in estrous cyclicity, secretion of hormones, and hypothalamic neural activity (21-24). The pattern of estrous cycles become irregular, the release of luteinizing hormone and prolactin becomes altered, and the catecholamine release from the hypothalamus is changed leading to development of mammary tumors at a later stage. We have unpublished evidence that there are significant changes in sympathetic noradrenergic activity in old female rats that may be responsible for suppressed immunological responses commonly observed in old rats. The results from the present study indicate that initiation of deprenyl

treatment in middle-aged female rats partially arrests the development of tumors by improving the neuroendocrine-immune interactions. Previously, we have demonstrated that deprenyl prevents the development of tumors young rats with carcinogen-induced mammary tumors accompanied by augmentation of hypothalamic dopaminergic activity (20). Several studies from our laboratory have provided evidence for the improvement in immunocompetence in young and old male rats, young and old rats with mammary tumors, and in old female rats (25-31). Further studies are essential to understand the mechanism(s) of action of deprenyl in preventing the development of tumors.

Key Research Accomplishments

- Deprenyl, a monoamine oxidase-B inhibitor, is an effective drug in arresting tumor growth in young rats that had carcinogen-induced tumors and in old rats that spontaneously developed mammary tumors
- The inhibition of tumor growth is achieved by enhancement of dopaminergic activity in the hypothalamus that also regulates prolactin secretion from anterior pituitary.
- Immune functions especially, IFN- γ production and the percentage of CD8+ T cells in spleen were higher in deprenyl-treated rats with spontaneously developing tumors and IL-2, IFN- γ production and NK cell activity were enhanced by deprenyl treatment in the spleens of young rats with carcinogen-induced tumors.
- Splenic NE concentration was also higher indicating that neural-immune interactions are also important in determining the degree of tumorigenesis.
- Deprenyl significantly inhibited the growth of breast cancer cells when added directly to cultures of estrogen receptor positive breast cancer cell lines whereas it had no effect on estrogen receptor negative breast cancer cell lines.
- Initiation of deprenyl treatment of middle-aged female rats (without tumors) inhibited the growth of mammary tumors at a later stage accompanied by enhancement of splenic NK cell activity and proliferation of lymphocytes in lymph nodes of deprenyl-treated rats.

Reportable Outcomes

Manuscripts, Abstracts, and Presentations.

1. ThyagaRajan S, Madden KS, Bellinger DL, Felten SY, Felten DL. Sympathetic modulation of immunity in aging. In: Oomura Y, Hori T, eds. Brain and Biodefence, Japan Scientific Societies Press, Tokyo and Karger, Switzerland, 1998: 27-38.
2. ThyagaRajan S, Madden KS, Felten SY, Felten DL. Inhibition of tumor growth by L-deprenyl involves neural-immune interactions in rats with spontaneously developing mammary tumors. *Anticancer Res.*, 19:5023-5028, 1999.
3. ThyagaRajan S, Madden KS, Felten SY, Felten DL. Anti-tumor effect of L-deprenyl is associated with enhanced central and peripheral neurotransmission and immune function in rats with carcinogen-induced mammary tumors. *J. Neuroimmunol*, 109: 95-104, 2000.
4. Felten DL. Neural influence on immune responses: underlying suppositions and basic principles of neural-immune signaling. *Prog Brain Res* 122:381-389, 2000.
5. ThyagaRajan S, Madden KS, Felten SY, Felten DL. Inhibitory effect of deprenyl on tumor growth involves enhancement of cell-mediated immunity in rats with carcinogen-induced mammary tumors. University of Rochester Cancer Center Second Annual Scientific Symposium (Abstract). 1997.

6. ThyagaRajan S, Madden KS, Felten DL. L-deprenyl-induced inhibition of mammary tumor growth is associated with altered neural activity and immune function in rats. Department of Defense Breast Cancer Research Program meeting, Era of Hope, Proceedings, volume II, 682, 2000.

7. ThyagaRajan S, Felten DL. Deprenyl: A modulator of neuroendocrine-immune interactions in breast cancer. An abstract submitted for the Cancer Detection and Prevention meeting to be held in October 2000.

Funding applied for based on work supported by this grant

PENDING

1. Felten, D. L.

BC001124	03/01/01 – 02/29/04	10%
Breast Cancer Research Program, U. S. Army \$299,967 (Total Direct Costs)		
Deprenyl and Protection against Breast Cancer Metastasis		

The major goal of this project is to investigate the role of deprenyl in preventing metastasis of tumors through the involvement of neuroendocrine-immune system.

2. ThyagaRajan, S.

BC001131	03/01/01 – 02/29/04	63%
Breast Cancer Research Program, U. S. Army \$143,858 (Total Direct Costs)		
Neural-Immune Interactions in Mammary Tumorigenesis		

The major goal of this project is to investigate the differences in neuroendocrine-immune interactions in three different rat strains with mammary tumors. This grant is for salary support.

3. ThyagaRajan, S.

BC001017	03/01/01 – 02/29/04	0%
Breast Cancer Research Program, U. S. Army \$299,951 (Total Direct Costs)		
Neuroendocrine-Immune Interactions in Breast Cancer		

The major goal of this project is to investigate the differences in neuroendocrine-immune interactions in three different rat strains with mammary tumors.

4. ThyagaRajan, S.

Michigan	01/01/01 – 12/31/03	20%
Life Sciences Corridor \$169,543 (Total Direct Costs)		
Aging, Neuroimmune Interactions, and Dietary Restriction		

The major goal of this project is to investigate the effects of dietary restriction on growth and development of tumors in rats with mammary tumors.

Employment/Research Opportunities

David L. Felten, M. D., Ph. D.

S. ThyagaRajan, Ph. D.

Lily Tran, B. S.

Kelley S. Madden, Ph. D.
Suzanne Y. Stevens, Ph. D.

Conclusions

The results from our studies demonstrate that breast cancer affects the functioning of neuroendocrine-immune interactions and deprenyl can be used to reverse such deficits.

Deprenyl treatment of rats with chemically-induced and spontaneously developing mammary tumors inhibited the development and growth of tumors, increased dopamine neurotransmission in specific brain regions, and increased splenic norepinephrine (NE) concentration. Furthermore, in tumor-bearing animals deprenyl treatment also enhanced interleukin -2 (IL-2) and interferon- γ (IFN- γ) production, and natural killer (NK) cell activity, important measures of anti-tumor immunity. These results from the present study suggest that both the central and peripheral nervous systems are involved in regulating mammary tumor growth possibly through the immune system. Stress and depression are common in cancer patients. The effects of stress and depression are mediated through hormones and neurotransmitters that in turn, modulate immune functions. The results from this project will help us understand the extent of neural-immune interactions in mammary tumorigenesis and provide impetus for further research on the role of central and peripheral nervous systems during metastasis and stress.

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Appendices

Manuscripts, Abstracts, and Presentations.

1. ThyagaRajan S, Madden KS, Bellinger DL, Felten SY, Felten DL. Sympathetic modulation of immunity in aging. In: Oomura Y, Hori T, eds. *Brain and Biodefence*, Japan Scientific Societies Press, Tokyo and Karger, Switzerland, 1998: 27-38.
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Anti-tumor effect of L-deprenyl is associated with enhanced central and peripheral neurotransmission and immune reactivity in rats with carcinogen-induced mammary tumors

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Abstract

L-Deprenyl, a monoamine oxidase-B (MAO-B) inhibitor, has previously been shown to improve immune responses and restore noradrenergic (NA) nerve fibers in the spleen of old rats. In tumor-bearing rats, L-deprenyl inhibited tumor incidence and enhanced tuberoindubular dopaminergic (TIDA) neurotransmission in the hypothalamus. The aim of the present study was to investigate whether alterations in sympathetic NA activity and cellular immune responses in the spleen, and TIDA activity in the hypothalamus, accompany deprenyl-induced regression of 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced mammary tumors. Rats with DMBA-induced mammary tumors were treated with 0, 2.5 mg, or 5.0 mg/kg body weight of deprenyl daily for 13 weeks. Saline-treated tumor-bearing rats exhibited reduced splenic IL-2 and IFN- γ levels, and lowered splenic norepinephrine (NE) concentration and hypothalamic dopaminergic activity, compared to rats without tumors. In contrast, treatment with 2.5 mg/kg and 5.0 mg/kg of deprenyl reduced the number and size of mammary tumors. Deprenyl-induced tumor regression was accompanied by increased immune measures in the spleen, including enhanced IL-2 and IFN- γ production, and NK cell activity. Neural measures enhanced by deprenyl included NE concentration in the spleen and TIDA neuronal activity in the hypothalamus. These results suggest that (1) mammary tumorigenesis is associated with the inhibition of sympathetic NA activity in the spleen, TIDA activity in the hypothalamus, and cell-mediated immunity, and (2) reversal of the inhibition of catecholaminergic neuronal activities of the central nervous system and peripheral nervous system by deprenyl may enhance anti-tumor immunity. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Catecholamines; Cytokines; Hypothalamus; NK cell activity; Selegiline; Tumor immunity

1. Introduction

Mammary tumorigenesis in rodents, like breast cancer in women, is a complex process involving the neuroendocrine system and the immune system (Dickson and Lippman, 1992; Meites, 1980; Souberbielle and Dalglish, 1994). Hormones, especially prolactin (PRL) from the anterior pituitary and estrogen (E) from the ovaries, under the influence of hypothalamic neurotransmitters and neuropeptides, control the development and growth of mammary tumors. Hypothalamic neurotransmitters and neuropeptides either inhibit or stimulate PRL secretion to control tumor growth. The tuberoindubular dopaminergic (TIDA) system in the medial basal hypothalamus (MBH) is an

important dopaminergic system that regulates PRL secretion through the release of dopamine (DA), the primary neurotransmitter that inhibits PRL secretion (Meites, 1980). Manipulation of the TIDA system has been reported to induce either a reduction or an increase in PRL secretion, leading to inhibition or acceleration of tumor growth, respectively (Meites, 1980; Quadri et al., 1973). L-Deprenyl is a monoamine oxidase-B (MAO-B) inhibitor that prevents enzymatic breakdown of DA, norepinephrine and serotonin (5-HT) (Knoll, 1980). L-Deprenyl has been used in the treatment of Parkinson's disease because of its ability to ameliorate the severity of symptoms through enhancement of nigrostriatal dopaminergic activity and has been shown to improve the cognitive functions in patients with Alzheimer's disease, presumably through an increase in nerve growth factor synthesis and cholinergic activity (Tariot et al., 1987; Tetrad and Langston, 1989). In rodents, deprenyl treatment was shown to stimulate growth

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factor synthesis and to elevate the activities of superoxide dismutase (SOD) and catalase in the brain, suggesting that the deprenyl-induced therapeutic benefits in the treatment of human neurodegenerative diseases may not be limited to inhibition of MAO alone (Carrillo et al., 1994; Seinuk et al., 1994). Treatment with deprenyl leads to increased dopaminergic activity in the MBH and reduced PRL secretion from the anterior pituitary (ThyagaRajan et al., 1995). In young rats with 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced mammary tumors, chronic deprenyl treatment reduced tumor growth and burden, and treatment of old female rats with deprenyl decreased the incidence of spontaneously occurring mammary and pituitary tumors (ThyagaRajan et al., 1995, 1998a; ThyagaRajan and Quadri, 1999). Presumably, the anti-tumor effect of deprenyl was mediated by the decrease in PRL secretion and thus, removal of a potent growth signal for tumors (ThyagaRajan et al., 1995, 1998a; ThyagaRajan and Quadri, 1999). However, evidence from our laboratory suggests that other mechanisms may contribute to the deprenyl-induced inhibition of tumor growth.

A functional relationship exists between sympathetic noradrenergic (NA) nerve fibers and cells of the immune system in lymphoid organs, as demonstrated by the presence of neuro-effector junctions between NA nerve fibers and lymphocytes, localization of adrenoceptors on many subsets of immunocytes, and alterations in immune functions following pharmacological manipulation of the sympathetic nervous system (Madden et al., 1995). In old rats, a conspicuous decline in NA innervation and NE content is observed in the splenic white pulp accompanied by a loss of T cell-mediated immune responses, including reduced T cell proliferation and IL-2 production by antigen- and mitogen-stimulated lymphocytes and lower NK cell activity (Bellinger et al., 1992; Pahlavani and Richardson, 1996). Similar reductions in immune reactivity have been reported in thymus and spleen of tumor-bearing rats (Gallo et al., 1993; Souberbielle and Dalglish, 1994). Recent studies from our laboratory have demonstrated that administration of deprenyl to old rats not only reversed the age-related decline in splenic NA innervation, but also enhanced splenic Con A-induced IL-2 production and NK cell activity (ThyagaRajan et al., 1998b). These results suggest that the anti-tumor effects of deprenyl may be mediated through enhancement of immune function, and these alterations in immune function may be driven by changes in the sympathetic nervous system.

The present study was conducted to investigate (1) how mammary tumorigenesis alters NE levels in the spleen and monoamine metabolism in the MBH; and (2) to determine if the anti-tumor effects of deprenyl are associated with alterations in sympathetic nervous system in the spleen and catecholaminergic activity in the MBH, and reversal of the immune dysfunction in rats with DMBA-induced mam-

mary tumors. The results suggest that the anti-tumor effects of deprenyl may be mediated through interactions between the nervous system and the immune system.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats (28- to 29-days-old) were purchased from Charles River Laboratories, Kingston, NY, USA and housed (5 animals/cage) in a temperature-controlled and light-controlled (12:12 h light–dark cycle) animal room. All animals received food and water *ad libitum*. At the age of 50–55 days, each animal received a single dose of 10 mg of DMBA (Sigma, St. Louis, MO, USA) dissolved in 1 ml of peanut oil by gastric intubation and housed individually. A separate group of animals (oil; $n=9$) received 1 ml of peanut oil alone. The animals were palpated for the development of tumors every week. Most of the rats developed mammary tumors within 2–3 months of DMBA administration. None of the rats that received peanut oil developed mammary tumors.

2.2. Treatment

After the appearance of tumors (1–2 cm in diameter), the rats were randomly divided into four different groups. Each group received either saline ($n=12$), 2.5 mg ($n=10$) or 5.0 mg ($n=15$) of deprenyl/kg BW/day intraperitoneally (i.p.) for 13 weeks. *R*-(–)-Deprenyl hydrochloride (α -deprenyl) was purchased from RBI, Natick, MA, USA. Tumor diameter and tumor number were measured every week throughout the treatment period. Tumor diameter was calculated by averaging two perpendicular diameters measured by vernier calipers. Percent change in tumor diameter was calculated using the equation (average diameter in $\text{cm}_{\text{week } n}$ – average diameter in $\text{cm}_{\text{week } 0}$) $\times 100$. At the end of the treatment period, the animals were sacrificed, and the MBH was rapidly removed and frozen immediately on dry ice. Spleens were removed aseptically and cut into three blocks at the hilus; two out of three blocks were frozen on dry ice, and stored at -80°C until further analysis for high-performance liquid chromatography with electrochemical detection (HPLC–ED). There is extensive distribution of NA nerve fibers in the hilar region as the nerve bundles enter the spleen at the hilus along with the splenic artery and are then distributed to other regions of the spleen (Bellinger et al., 1993). The third part of the spleen was used for immunological assays including NK cell activity, IFN- γ production, IL-2 production, Con A-induced T lymphocyte proliferation and flow cytometry.

2.3. Lymphocyte preparation

Lymphocytes from the spleen were prepared as described previously (Madden et al., 1994; ThyagaRajan et al., 1998b). Cells were resuspended to the desired concentration in RPMI 1640 medium supplemented with 5% fetal calf serum (Sigma), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.01 mM nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, 24 mM sodium hydrogencarbonate, and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) for in vitro culture.

2.4. NK cell activity

NK cell activity was assessed using the NK-sensitive lymphoma YAC-1 passaged in vitro. YAC-1 cells in log phase growth were incubated with 100 μ Ci of $\text{Na}_2^{51}\text{CrO}_4$ (DuPont NEN, Boston, MA, USA) at 37°C for 90 min. The cells were washed three times and adjusted to 10^5 cells/ml. Spleen cells ratios were mixed with 10^4 ^{51}Cr -labeled YAC-1 cells at varying effector to target (E:T) ratios in round-bottom 96-well tissue culture plates (Falcon, Becton Dickinson, Oxnard, CA, USA) in triplicate in a volume of 200 μ l. Spontaneous release was determined by incubating 10^4 ^{51}Cr -labeled YAC-1 cells with complete RPMI alone. Maximum release was determined by adding 1% Triton X-100 to 10^4 ^{51}Cr -labeled YAC-1 cells. The plates were then centrifuged at 200 g for 5 min and incubated for 4 h at 37°C in a CO_2 -humidified atmosphere. The plates were centrifuged at 500 g for 5 min at 4°C, and 100 μ l of supernatant was removed from each well, and radioactivity was counted in a gamma counter. Cytotoxic activity was expressed as percent lysis, determined by the equation (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm) $\times 100$.

2.5. Assay for IL-2 and IFN- γ production

Lymphocytes (2×10^5 cells/well) were incubated with either medium alone or 1.25 μ g/ml of Con A in 24-well tissue culture plates (Falcon, Becton Dickinson). After 24 h of culture, 1 ml of supernatant was removed from each well and stored at -20°C until assayed for cytokine content.

To measure IL-2, supernatants collected after the incubation period were tested for the ability to support the growth of the IL-2 dependent cell line, CTLL-2. Supernatants from individual wells were serially diluted and incubated with 4×10^3 CTLL-2 cells in a total volume of 100 μ l for 40 h. Cell growth was then determined using a colorimetric assay as described (Madden et al., 1994). A 10- μ l volume of 5.0 mg/ml 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) in HBSS (Hanks Balanced Salt Solution) was added to each well.

After incubating for 4 h at 37°C, 100 μ l of 0.04 N HCl in isopropanol was added to dissolve the colored precipitate. Absorbance was measured with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Background values (CTLL-2 cells incubated with medium only) were subtracted automatically from each value. These values were compared to recombinant murine IL-2 (Genzyme, Cambridge, MA, USA).

IFN- γ levels in supernatants were determined by enzyme-linked immunosorbent assay (ELISA). ELISA plates (Corning, Corning, NY, USA) were coated overnight at 4°C with purified anti-rat IFN- γ polyclonal Ab (1 μ g/ml; Biosource International, Camarillo, CA, USA) in 0.1 M Na_2HPO_4 buffer (pH 9.0). In between steps, plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tween). Plates were then blocked for 2 h with PBS-10% fetal equine serum (FES) at room temperature. Recombinant rat IFN- γ (Biosource) or samples serially diluted in culture media were added to plates in triplicate and incubated overnight at 4°C. Biotin-conjugated anti-rat IFN- γ (0.5 μ g/ml; Biosource), diluted in PBS-10% FES, was added and the plates were incubated at room temperature for 1 h. Avidin-peroxidase (Sigma), diluted 1:400 in PBS-10% FES, was added to the plates, and incubated for 30 min at room temperature. In the final step, substrate ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; Sigma) containing 0.03% hydrogen peroxide was added to the plates and incubated for 30 min at room temperature. Absorbance at 405 nm was measured with a microplate reader (Bio-Tek Instruments) after 30 min. The amount of IFN- γ in samples was determined by extrapolation to the standard curve.

2.6. Con A-induced proliferation

Spleen cells, 2×10^5 cells/well, were cultured in triplicate with either medium alone or varying concentrations of Con A (Calbiochem-Behring, La Jolla, CA, USA), in 96-well, flat bottom tissue culture plates (Falcon), and maintained for 3 days at 37°C in 5% CO_2 -humidified incubator. [^3H]Thymidine (0.5 μ Ci/10 μ l; 5 Ci/mmol; DuPont NEN) was added for the final 18 h of culture. Cells were harvested onto glass fiber filter paper (Whatman, Clifton, NJ, USA) with a cell harvester (Skatron). The dried filters were placed in scintillation fluid (Biosafe II, RPI, Mount Prospect, IL, USA), and radioactivity determined with a liquid scintillation counter (LKB, Wallac, Finland).

2.7. Flow cytometric analysis

Spleen cells were washed in PBS containing 2% bovine serum albumin (BSA) and 0.02% azide (flow wash). Fluorescein-conjugated anti-rat sIgM (clone G53-238, di-

luted 1:40; Pharmingen) and phycoerythrin-conjugated anti-NKR-P1A (an NK cell marker, clone 10/78, diluted 1:40; Pharmingen) or fluorescein-conjugated anti-rat CD8 (clone OX-8, diluted 1:40; Pharmingen) and phycoerythrin-conjugated anti-CD4 (clone OX-35, diluted 1:20; Pharmingen) were added to 2×10^6 cells and incubated at 4°C for 30 min. Cells incubated with flow wash alone were included to determine autofluorescence. Following this incubation, cells were washed twice in flow wash, fixed in PBS containing 1% paraformaldehyde, and stored in the dark for no longer than 2 weeks at 4°C prior to analysis. Two-color fluorescence was analyzed with an Elite flow cytometer (Coulter Electronics, Hialeah, FL, USA), equipped with an argon laser at 15 mW and excitation wavelength of 488 nm.

2.8. HPLC–ED

Prior to analysis by HPLC–ED, NE in the spleen was extracted with alumina. Tissues were homogenized in 0.1 M of HClO_4 containing 0.25 μM of 3,4-dihydroxybenzylamine (DHBA) as the internal standard and were centrifuged at 1000 g for 5 min. The supernatants were used for the aluminum oxide extraction and the pellets were saved for protein assay (Bio-Rad assay kit).

For the estimation of monoamines in the MBH, a volume of 200 μl of HClO_4 containing 0.25 μM of DHBA was added as the internal standard to the tubes containing MBH. The tissues were sonicated and centrifuged for 2 min at 1000 g. The supernatants were stored at -80°C until analyzed for the concentrations of NE, DA, 5-HT, and their metabolites by HPLC–ED. Pellets were stored for the measurement of protein concentrations.

At the time of HPLC–ED analysis, samples were loaded onto a Waters 717plus autosampler (Waters, Milford, MA, USA) and NE concentration was measured using HPLC–ED as described previously (ThyagaRajan et al., 1998a,b). NE concentration in the spleen was expressed as pmol/mg protein and pmol/mg wet weight of the tissue. NE content in the whole spleen was calculated using NE concentration/mg wet weight in the combined hilar and end region of the spleen (ThyagaRajan et al., 1998b). The neurotransmitter concentrations in the MBH were expressed in terms of pmol/mg protein.

2.9. Statistical analysis

The data were analyzed by analysis of variance (ANOVA). NK cell activity and Con A-induced proliferation were analyzed using ANOVA with *E:T* ratio and Con A concentration as repeated measures. Parameters that attained significance following ANOVA ($P < 0.05$) were further analyzed by Fisher's least-significant difference test.

3. Results

Animals from each group were sacrificed at two different intervals because of tumor development and US National Institutes of Health (NIH) guidelines on tumor growth. Due to a lack of statistically significant difference between the data based on tissue collection, the following results are from the pooled data from the two time-points. The tumor growth and immune functions in a group of rats ($n=4$) treated with 0.25 mg/kg deprenyl were similar to saline-treated tumor-bearing rats in a preliminary study and therefore, was not included in the present study.

Mammary tumor growth during the 13-week period of daily i.p. deprenyl administration is shown in Fig. 1. Animals treated with saline showed a consistent increase in average tumor diameter, almost doubling in size by the end of the treatment period. In contrast, treatment with 2.5 mg/kg and 5.0 mg/kg deprenyl inhibited ($P < 0.05$) tumor growth in comparison to the saline group. Three animals in the saline-treated group had excessive tumor growth and died due to excoriation of tumors, whereas none of the animals died in the deprenyl-treated groups. Animals that received no DMBA (peanut oil alone) exhibited no tumors.

All animals had one or two DMBA-induced tumors at the beginning of the treatment period (Fig. 2). In the saline group, a progressive increase in the average number of tumors to $2.6 \pm 0.3/\text{rat}$ was observed at the end of the 13-week treatment period. Treatment with 2.5 mg/kg ($1.8 \pm 0.4/\text{rat}$) and 5.0 mg/kg ($1.5 \pm 0.2/\text{rat}$) deprenyl significantly prevented the increase in tumor number during the final 2 weeks and from the ninth week of treatment period, respectively.

To assess T cell function, IL-2 and IFN- γ production

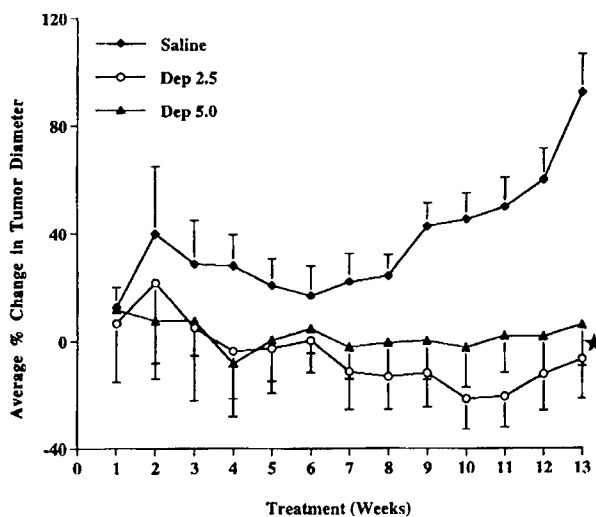


Fig. 1. Effects of deprenyl on tumor diameter in rats with DMBA-induced mammary tumors. Young Sprague–Dawley female rats received 10 mg of DMBA in 1 ml of peanut oil by gastric intubation. Animals were treated with saline or deprenyl for 13 weeks after the development of tumors. *Significantly ($P < 0.05$) different from the saline group.

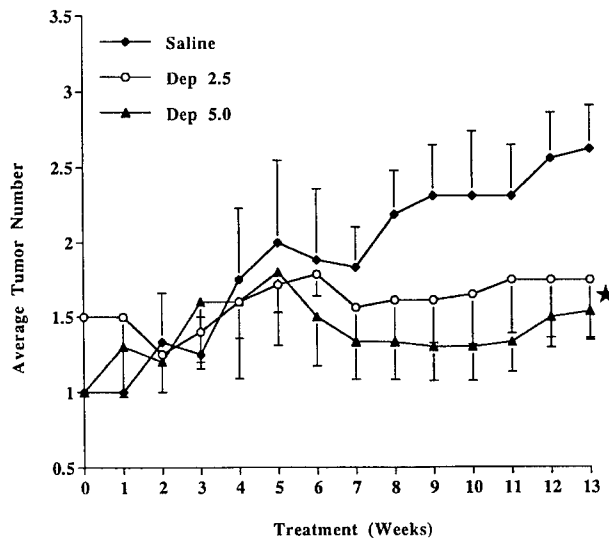


Fig. 2. Effects of deprenyl on tumor number in rats with DMBA-induced mammary tumors. *Significantly ($P < 0.05$) different from the saline group.

were measured in supernatants obtained from Con A-stimulated splenocytes. Compared to animals with no tumors, IL-2 production was significantly reduced in the saline group (Fig. 3). Treatment with 2.5 mg/kg and 5.0 mg/kg deprenyl significantly increased IL-2 production in comparison to the saline group. Spleen cell IFN- γ production also was decreased in saline-treated tumor-bearing rats (Fig. 4). In contrast, treatment with 5.0 mg/kg deprenyl increased splenic IFN- γ production. Con A-induced T cell proliferation was decreased in saline- and

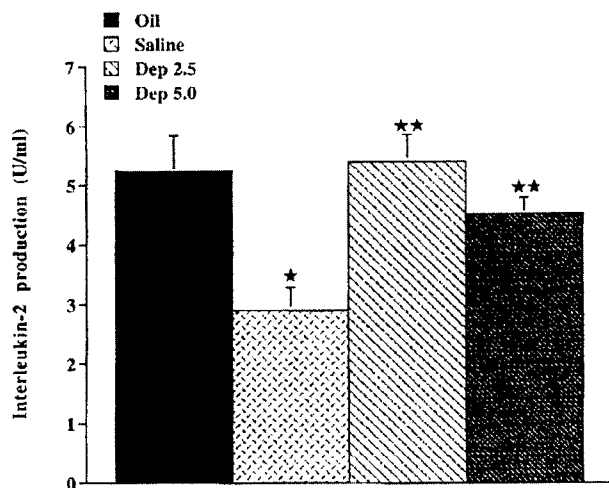


Fig. 3. IL-2 production by spleen cells from rats with DMBA-induced mammary tumors after 13 weeks of treatment with 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl. Spleen cells were incubated with 1.25 μ g/ml of Con A for 24 h. Supernatants were cultured with the IL-2 dependent cell line, CTLL and cell growth was measured colorimetrically. *Significantly ($P < 0.05$) different from oil group. **Significantly ($P < 0.05$) different from saline group.

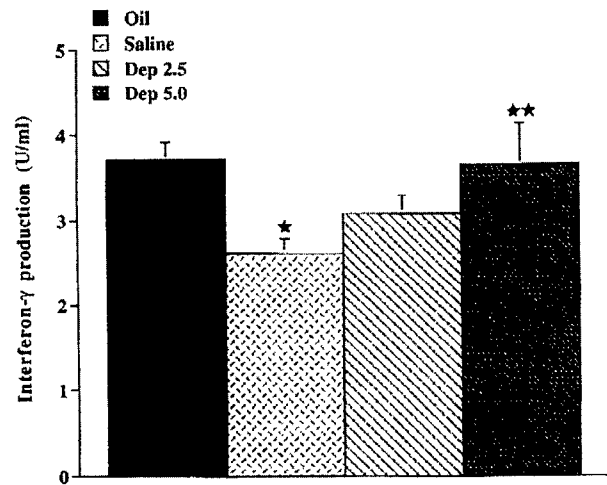


Fig. 4. IFN- γ production by spleen cells from rats with DMBA-induced mammary tumors after 13 weeks of treatment with 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl. Spleen cells were incubated with 1.25 μ g/ml of Con A for 24 h. Supernatants were used for measurement of IFN- γ production by ELISA. *Significantly ($P < 0.05$) different from oil group. **Significantly ($P < 0.05$) different from saline group.

deprenyl-treated tumor-bearing rats in comparison to animals with no tumors (Fig. 5). Flow cytometric analysis of splenic T cell populations revealed a significant reduction in CD8+ T cells in tumor-bearing rats (Table 1). Deprenyl induced no alterations in the percentage of sIgM+ B cells or CD4+ T cells.

NK cell activity analyzed by ANOVA revealed a three-

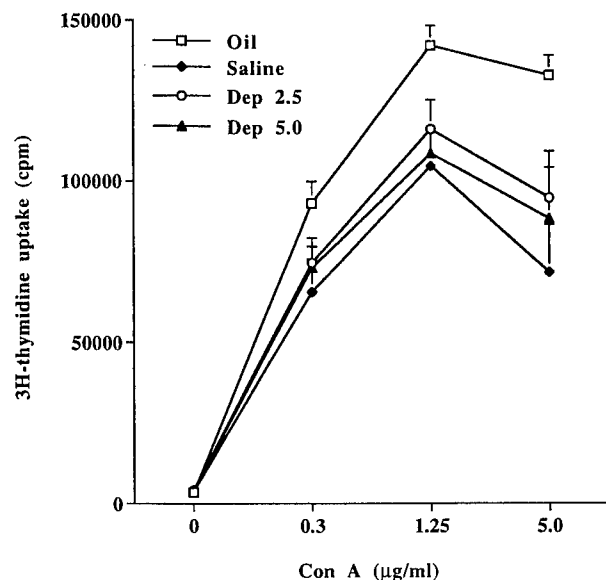


Fig. 5. Con A-induced T lymphocyte proliferation by spleen cells from rats with DMBA-induced mammary tumors after 13 weeks of treatment with 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl. Spleen cells were incubated with 0, 0.3, 1.25, or 5 μ g/ml of Con A for 72 h. Proliferation of T lymphocytes was reduced in saline- and deprenyl-treated rats in comparison to rats that received oil alone.

Table 1

Spleen lymphocyte population from rats with carcinogen-induced mammary tumors*

Group	% sIgM+	% CD4+	% CD8+	% NK+
Oil	36.7±1.2	44.9±1.0	15.7±0.8	3.9±0.4
Saline	40.1±1.6	43.1±1.4	12.0±0.9*	3.8±0.4
Dep 2.5	35.7±1.8	39.0±2.1	11.8±0.7*	3.8±0.3
Dep 5.0	36.4±2.0	40.0±2.6	10.9±0.9*	3.1±0.7

* Rats were treated with 0, 2.5 mg, or 5.0 mg/kg BW of deprenyl daily for 13 weeks. All values are mean±S.E.M.

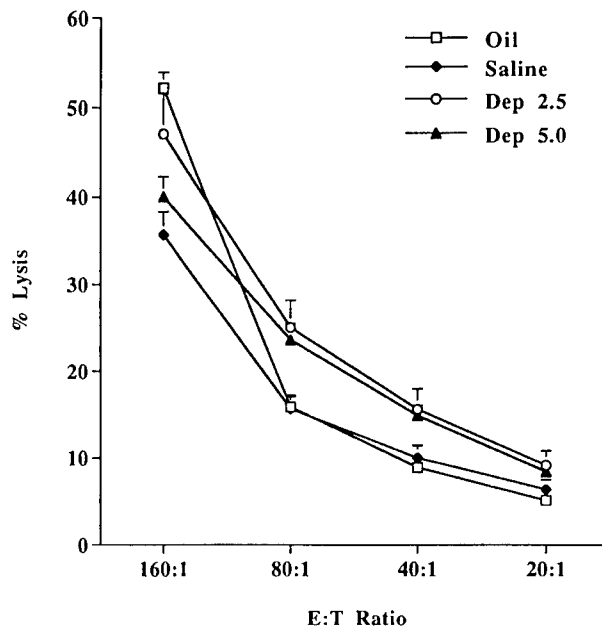
*Significantly ($P<0.001$) different from oil.

Fig. 6. Splenic NK cell activity in rats with DMBA-induced mammary tumors after 13 weeks of treatment with 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl. Animals in oil group received 1 ml of peanut oil alone. Spleen cells from saline and 5.0 mg/kg deprenyl-treated rats showed reduced NK cell activity at a higher $E:T$ ratio (160:1) in comparison to rats in oil group. Daily administration of 2.5 mg/kg and 5.0 mg/kg deprenyl enhanced NK cell activity relative to oil and saline-treated rats at 80:1 and 40:1 $E:T$ ratios.

way interaction between treatment period, drug treatment and $E:T$ ratio (Fig. 6). NK cell activity at a higher ratio (160:1) was lower in tumor-bearing rats treated with saline and 5.0 mg/kg deprenyl compared to animals without tumors, but it was higher in rats treated with 2.5 mg/kg deprenyl in comparison to saline-treated rats. NK cell activity was significantly increased at lower $E:T$ ratios (80:1 and 40:1) in animals that received daily treatment of 2.5 mg/kg and 5.0 mg/kg deprenyl compared to oil and saline-treated groups. The deprenyl-induced increase in NK cell activity was not associated with alterations in the percentage of NK cells (Table 1).

In comparison to rats without tumors, the concentration of NE (per mg protein and per mg wet weight) was decreased significantly ($P<0.05$) in both the hilar and end regions of the spleens from tumor-bearing rats treated with saline (Table 2). In contrast, splenic NE (per mg protein) was not significantly reduced in animals treated with 2.5 mg/kg and 5.0 mg/kg deprenyl. Administration of 5.0 mg/kg deprenyl for 13 weeks also significantly increased NE concentration (pmol/mg protein) in the hilar region of the spleen relative to the saline group. Calculated for the whole spleen, a decline in NE content was observed in deprenyl-treated rats in comparison to rats without tumors except in the 5.0 mg/kg group. There was no significant difference in the wet weight of spleen due to treatment with deprenyl.

The concentration of the DA metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), was higher in the MBH of saline-treated rats in comparison to rats with no tumors (oil; Table 3) and deprenyl treatment significantly ($P<0.01$) reduced DOPAC concentration relative to saline-treated rats. There was no statistically significant changes in the concentration of DA with saline or deprenyl treatment in tumor-bearing animals. However, treatment with 2.5 mg/kg and 5.0 mg/kg deprenyl showed a tendency to increase DA concentration ($P=0.06$). No alterations in NE, 5-HT, or 5-hydroxyindoleacetic acid (5-HIAA; 5-HT metabolite) concentrations were observed in the MBH of saline-treated tumor-bearing rats, compared to the rats with no tumors. However, NE and 5-HT

Table 2

Effects of deprenyl treatment on splenic norepinephrine (NE) concentration in rats with DMBA-induced mammary tumors*

Group	Whole spleen weight (g)	NE concentration in spleen				NE content in whole spleen (pmol/mg wet weight)
		Hilar region		End region		
		pmol/mg protein	pmol/mg wet weight	pmol/mg protein	pmol/mg wet weight	
Oil	0.53±0.02	14.5±1.8	696.1±63.1	19.1±1.4	777.4±44.4	3333.6±272.4
Saline	0.75±0.08	8.6±1.4*	385.9±75.6*	10.6±1.8*	457.8±68.5*	1802.9±279.4*
Dep 2.5	0.70±0.08	9.8±1.3	438.2±53.7*	13.2±1.4	584.4±71.5	2361.8±289.2*
Dep 5.0	1.02±0.21	13.4±1.7**	553.6±84.5	15.8±2.8	623.9±96.2	2525.6±378.2

* Rats were treated with 0, 2.5 mg, or 5.0 mg/kg BW of deprenyl daily for 13 weeks. All values are mean±S.E.M.

*Significantly ($P<0.001$) different from oil.**Significantly ($P<0.05$) different from saline.

Table 3

Effects of deprenyl treatment on catecholamines, indoleamine, and their metabolites in the medial basal hypothalamus of rats with carcinogen-induced mammary tumors^a

Group	NE	DOPAC	DA	5-HIAA	5-HT
Oil	173.5±10.2	14.3±2.5	66.1±9.7	42.7±3.7	77.1±5.3
Saline	203.2±11.7	25.2±4.1*	72.9±3.2	58.4±5.4	73.3±2.9
Dep 2.5	306.6±15.5§	9.5±1.1**	82.5±5.1	47.9±2.8	107.5±5.9§
Dep 5.0	347.5±32.4§	10.5±1.8**	100.1±11.9	46.6±4.6	118.0±10.4§

^a Rats were treated with 0, 2.5 mg, or 5.0 mg/kg BW of deprenyl daily for 13 weeks. All values are mean±S.E.M.

*Significantly ($P<0.01$) different from oil.

**Significantly ($P<0.01$) different from saline.

§Significantly ($P<0.01$) different from oil and saline.

concentrations were significantly ($P<0.01$) higher in the MBH of rats that received 2.5 mg/kg and 5.0 mg/kg deprenyl relative to saline-treated rats.

4. Discussion

A decline in cell-mediated and humoral immunity is associated with mammary tumorigenicity in rodents (Cawein and Sydnor, 1968; Kearney and Hughes, 1970; Souberbielle and Dalglish, 1994). The results from the present study demonstrate that the inhibition of splenic immune reactivity was associated with reduced NE concentration in the spleen and increased levels of DOPAC, a DA metabolite, in the MBH of DMBA-induced mammary tumors. Treatment of tumor-bearing rats with deprenyl daily for 13 weeks inhibited tumor growth and development, and reversed the alterations in splenic NE concentration, immune responses, and hypothalamic dopaminergic activity suggesting that the anti-tumor effects of deprenyl can be mediated by improved immune functions, facilitated by changes in the central and peripheral nervous systems. These changes, together with its ability to modulate PRL secretion, indicate that deprenyl may act through both immune and neuroendocrine mechanisms to inhibit tumor growth.

In tumor-bearing saline-treated rats, we observed reduced splenic NK cell activity, and IL-2 and IFN- γ production. The reduction in NK cell activity in the saline-treated rats may be responsible for tumor progression since NK cells are necessary for tumor surveillance (Herberman and Holden, 1978). Lower levels of splenic IL-2 and IFN- γ production in saline-treated rats may have contributed to the reduction in NK cell activity because IL-2 and IFN- γ can enhance NK cell activity and arrest tumor growth (Souberbielle and Dalglish, 1994). The importance of IL-2 and IFN- γ in anti-tumor immunity was demonstrated by the ability of tumor cells transduced with viral vectors containing IL-2 gene or IFN- γ gene to suppress tumor growth (Addison et al., 1995; Watanabe, 1992). Deprenyl increased IL-2 and IFN- γ production, and NK cell activity in tumor-bearing animals. An increase in Con A-induced IFN- γ production was also observed in

deprenyl-treated rats with spontaneously developing tumors (ThyagaRajan et al., 1999b). The increase in splenic NK cell activity occurred in the absence of changes in the percentage of NK cells, indicating that NK cell activity per cell was increased by deprenyl treatment. A significant reduction in Con A-induced T lymphocyte proliferation occurred in the spleens of saline- and deprenyl-treated rats. Similarly, the percentage of CD8+ T cells decreased in tumor-bearing rats that was not reversed by deprenyl treatment. However, administration of deprenyl to rats with spontaneously occurring mammary tumors resulted in a moderate increase in the percentage of CD8+ T cells in the spleen (ThyagaRajan et al., 1999b). It is not known whether these changes reflect an actual reduction in T cell function or a redistribution of T lymphocytes to other lymphoid organs and/or to the tumor.

In the present study, development and growth of DMBA-induced tumors was associated with reduced splenic NE concentration. This alteration in NE may contribute to the reduced NK cell activity and cytokine production observed in these animals. Such a relationship is evident in other systems. Reduced T lymphocyte proliferation, IL-2 secretion, and NK cell activity is accompanied by diminished NA innervation in spleen and lymph nodes of old rats (Bellinger et al., 1992, 1993; Pahlavani and Richardson, 1996). Treatment of old male rats with deprenyl reversed the age-related decline of NA innervation in the spleen and also improved splenic NK cell activity and IL-2 production (ThyagaRajan et al., 1998b). Similarly, treatment of young male rats with deprenyl increased splenic NK cell activity, Con A-induced T lymphocyte proliferation, and NE content (ThyagaRajan et al., 1999a). In rats with spontaneously developing mammary tumors, deprenyl treatment suppressed tumor growth and increased splenic NE concentration accompanied by improvement in immune functions (ThyagaRajan et al., 1999b). In young mice, destruction of NA nerve terminals by chemical ablation with 6-hydroxydopamine results in depletion of NE in the periphery and diminished T cell-mediated immune responses, including delayed hypersensitivity, cytotoxic T lymphocyte activity, Con A-induced T cell proliferation, and IL-2 and IFN- γ production (Madden et al., 1989; Madden et al., 1994). In addition,

administration of α -methyl-*p*-tyrosine, an inhibitor of the rate-limiting enzyme for NE biosynthesis, tyrosine hydroxylase, decreased splenic NE concentration and NK cell activity in mice (Won and Lin, 1993). Together, this evidence suggests that reduced splenic NE can contribute to reduced immune function.

Another potential mechanism by which deprenyl may inhibit tumor growth is through enhanced central dopaminergic activity. Cessation of reproductive cycles and development of mammary and pituitary tumors associated with increased PRL secretion from the anterior pituitary is a common age-related phenomenon observed in female rats (Meites, 1980). Treatment of rats with agents that increase PRL secretion promotes the growth, while agents that decrease PRL secretion inhibit the growth, of both spontaneous and carcinogen-induced mammary tumors (Meites, 1980; Quadri et al., 1973; Welsch and Nagasawa, 1977). DA is one of the several neurotransmitters and neuropeptides responsible for the inhibition of PRL secretion. Previously, we have demonstrated that deprenyl treatment reduced the incidence of spontaneously occurring mammary and pituitary tumors in old female rats and suppressed the development and growth of DMBA-induced mammary tumors when given before and after DMBA (ThyagaRajan et al., 1995, 1998a; ThyagaRajan and Quadri, 1999). These effects on tumorigenesis were accompanied by a reduction in the metabolism of DA in the MBH; resulting in increased DA and lower serum levels of PRL (MohanKumar et al., 1994; ThyagaRajan et al., 1995, 1998a; ThyagaRajan and Quadri, 1999).

The deprenyl-induced alterations in DA metabolism presented here are consistent with the known anti-tumor properties of deprenyl. Deprenyl inhibits the enzymatic degradation of DA by MAO-B. This is indicated by a reduction in the concentration of DOPAC, a metabolite of DA, and the slight increase DA in the MBH of deprenyl-treated rats. Both measures indicate decreased breakdown of DA resulting in increased availability of DA for the inhibition of PRL secretion. The increase in DOPAC concentration in the MBH of saline-treated tumor-bearing rats is noteworthy because it suggests enhanced MAO activity in the MBH is associated with tumor growth. Although DA levels were not decreased in these animals, increased MAO activity can be indicative of reduced DA release into the anterior pituitary. TIDA neurons have no other mechanisms for DA clearance, such as autoreceptors and high-affinity DA transporters (Demarest and Moore, 1979; Gudelsky and Metzger, 1984; Westfall et al., 1983). Thus tumor-bearing animals exhibit reduced DA availability and enhanced PRL levels, leading to increased tumor growth. Serotonin is another neurotransmitter that regulates PRL level by stimulating its secretion from the anterior pituitary (Meites, 1980). In spite of an increase in 5-HT concentration in the MBH by deprenyl, tumor growth was suppressed suggesting that the inhibitory effect

of DA may have been stronger than the stimulatory effect of serotonin on PRL secretion.

Changes in central DA, 5-HT and NE may also influence tumor growth through regulation of anti-tumor immunity. Lesioning of the TIDA system in the MBH leads to tumor growth and suppressed NK cell activity (Bindoni et al., 1980; Forni et al., 1983). Anterior and posterior hypothalamic lesions have resulted in altered splenocyte and thymocyte numbers, decreased Con A-induced T lymphocyte proliferation, NK cell activity, and antibody production suggesting that these areas in the hypothalamus are involved in the stimulation of cell-mediated and humoral immunity (Felten et al., 1991). Retrograde tracing of neuronal pathways from the spleen to the central nervous system revealed a neuronal circuitry between the spleen and discrete areas in the spinal cord, brain stem, and the hypothalamus, demonstrating that direct neuronal pathways to the spleen may alter immune reactivity, and that rapid bi-directional communication between the spleen and central nervous system is possible (Cano et al., 1997).

Another mechanism by which deprenyl may exert its anti-tumor effect is through increased scavenging of free radicals by SOD and catalase. Diminution of SOD activity in mammary tumors and glutathione peroxidase activity in the spleen of tumor-bearing rats can elevate free-radical levels resulting in lymphocyte-induced angiogenesis within the tumor, and tumor progression (L'Abbe et al., 1989; Monte et al., 1994; Whiteside et al., 1983). Deprenyl administration to old rats has been reported to increase the activities of SOD and catalase in various regions of the brain (Carrillo et al., 1994). It is possible that deprenyl exerted similar improvement in the activities of antioxidant enzymes in peripheral lymphoid organs and within the tumor to inhibit tumor growth.

In summary, we have demonstrated that the neuroimmunoendocrine system is disrupted in rats with DMBA-induced mammary tumors. Deprenyl treatment may prevent mammary tumorigenesis by stimulating immune function, enhancing central catecholaminergic activity, and increasing peripheral splenic NE. Further studies are needed to determine the mechanism(s) of deprenyl-induced anti-tumor activity and the relative importance of the changes in immune function and central vs. peripheral neuronal activity in inhibiting mammary tumor growth. The results suggest that deprenyl may be beneficial in the treatment of breast cancer in women by preventing neuroimmunoendocrine dysfunction.

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Inhibition of Tumor Growth by L-Deprenyl Involves Neural-Immune Interactions in Rats with Spontaneously Developing Mammary Tumors

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Abstract. *L-deprenyl, a monoamine oxidase-B inhibitor, has been shown to reverse the age-related decline in sympathetic noradrenergic innervation and immune function in old rats and enhance T cell and NK cell activity in tumor-bearing rats. The objective of the present study was to examine whether deprenyl treatment of old female rats with mammary tumors could augment sympathetic nervous system and immune responses to inhibit the tumor growth. Female Sprague-Dawley rats with spontaneous mammary tumors were administered 0, 2.5 mg, or 5.0 mg/kg body weight (BW)/day deprenyl for i.p. 9 weeks. Tumor diameter, tumor number and body weight were measured throughout the treatment period. At the end of the treatment period, norepinephrine (NE) concentration, interferon- γ production (IFN- γ), Con A-induced T lymphocyte proliferation, and percentage of T and B lymphocytes and natural killer cells were measured in the spleen, and the concentrations of monoamines were measured in the medial basal hypothalamus. Relative to saline-treated controls, treatment with deprenyl reduced tumor growth, increased NE concentration, IFN- γ production and percentage of the CD8+ T lymphocytes in the spleen. In the medial basal hypothalamus, deprenyl treatment increased the concentrations of catecholamines and indoleamine. These results suggest that the anti-tumor effects of deprenyl on spontaneous rat mammary tumors may be achieved via neural-immune signaling in the spleen and medial basal hypothalamus.*

Autonomous development and growth of spontaneous mammary tumors in aging female rats is associated with an increase in prolactin (PRL) and a decrease in ovarian hormone secretion (1). PRL secretion from the anterior

pituitary is primarily regulated by the release of dopamine (DA) from the tuberoinfundibular dopaminergic (TIDA) system in the medial basal hypothalamus (MBH). Administration of agonists and antagonists to DA release promoted or suppressed the development and growth of tumors, respectively, suggesting that mammary tumorigenesis is dependent upon the availability of PRL (1, 2). In addition to these neuroendocrine changes, suppression of immune system function facilitates growth and metastasis of tumors. Tumor incidence and progression is determined, in part, by cytokines that regulate macrophage, T- and B-lymphocyte activation, and enhance the activities of natural killer (NK) cells and lymphokine-activated killer cells (3). The sympathetic nervous system through the release of norepinephrine (NE) can regulate cytokine production and other activities of the immune system (4). Thus, the sympathetic noradrenergic (NA) system may play a role in modulating tumor-specific immune responses.

L-deprenyl, a specific MAO-B inhibitor, suppressed development and growth of tumors by augmenting hypothalamic TIDA activity and lowering PRL secretion in rats with carcinogen-induced mammary tumors (5, 6). Prolonged administration of deprenyl to old female rats inhibited the incidence of spontaneously developing mammary tumors by increasing DA levels in the MBH and decreasing serum PRL (7). Deprenyl has been used in the treatment of human neurodegenerative disorders due to its ability to improve nigrostriatal dopaminergic activity in Parkinson's disease and enhance cholinergic neurotransmission in Alzheimer's disease (8-10). Furthermore, treatment of old rats with deprenyl reversed the age-related decline in NA innervation of the spleen and also increased NK cell activity and IL-2 production, indicating that deprenyl is capable of altering functional activities of both the nervous and immune systems (11-12). A recent study revealed similar enhancement in NK cell activity, IL-2 and IFN- γ production, sympathetic NA activity in the spleen, and TIDA activity in the MBH of deprenyl-treated rats with carcinogen-induced mammary tumors (unpublished data). The present study was

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Key Words: Selegiline, catecholaminess, spleen, medial basal hypothalamus, interferon- γ , lymphocytes.

conducted to examine whether deprenyl can inhibit tumor growth in intact old female rats with spontaneously developing mammary tumors. To investigate the extent of involvement of central and peripheral nervous systems and immune system in the suppression of tumor growth, we assessed immune function and NE concentration in the spleen, and the concentrations of NE, DA, and serotonin in the MBH of deprenyl-treated old female rats.

Materials and Methods

Animals. Female Sprague-Dawley rats (12 mo-old) were purchased from Charles River Laboratories, Kingston, NY and housed individually in a temperature-controlled and light-controlled (12:12 h light/dark cycle) animal room. All animals received food and water *ad libitum*. The animals were palpated for the presence of tumors at the time of arrival and thereafter every week for the development of tumors. No tumors were detected at the time of arrival. After 7-8 months, animals started developing mammary tumors.

Treatment. After the appearance of tumors (1-2 cms in diameter), the rats were randomly divided into three different groups that received either saline (n=10), 2.5 mg (n=12) or 5.0 mg (n=11) of deprenyl/kg BW/day i.p. for 9 weeks. R(-)-Deprenyl hydrochloride was purchased from RBI, Natick, MA. Tumor diameter, tumor number, and body weight were measured every week throughout the treatment period. Tumor diameter was calculated by averaging two perpendicular diameters measured by vernier calipers. Percent change in tumor diameter was calculated using the equation, (Average diameter in cms week n - Average diameter in cms week 0) X 100. At the end of the treatment period, the animals were sacrificed, and the MBH were rapidly removed and frozen immediately on dry ice. Spleens were removed aseptically and cut into three blocks; two out of three blocks were frozen on dry ice, and stored at -80°C until further analysis for high performance liquid chromatography with electrochemical detection (HPLC-EC). The third block of spleen was used for immunological assays including IFN- γ production, Con A-induced proliferation of T lymphocytes, and flow cytometry.

Lymphocyte preparation. Lymphocytes from the spleen were prepared as described previously (12, 13). Cells were resuspended to the desired concentration in RPMI 1640 medium supplemented with 5% fetal calf serum (Sigma), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.01 mM nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, 24 mM sodium bicarbonate, and 10 mM HEPES for *in vitro* culture.

Assay for IFN- γ production. Lymphocytes (2×10^5 cells/well) were incubated with either medium alone or 1.25 μ g/ml of Con A in 24-well tissue culture plates (Falcon, Becton Dickinson, Oxnard, CA). After 24 h of culture, 1 ml of supernatant was removed from each well and stored at -20°C until assayed for cytokine content.

IFN- γ levels in supernatants were determined by ELISA. ELISA plates (Corning, Corning, NY) were coated overnight at 4°C with purified anti-rat IFN- γ polyclonal Ab (1 μ g/ml; Biosource International, Camarillo, CA) in 0.1 M Na₂HPO₄ buffer (pH 9.0). In between steps, plates were washed with PBS containing 0.05% Tween-20 (PBS/Tween). Plates were then blocked for 2 h with PBS-10% fetal equine serum (FES) at room temperature. Recombinant rat IFN- γ (Biosource) or samples serially diluted in culture media were added to plates in triplicate and incubated overnight at 4°C. Biotin-conjugated anti-rat IFN- γ (0.5 μ g/ml; Biosource), diluted in PBS-10% FES, was added and the plates were incubated at room temperature for 1 h. Avidin-peroxidase (Sigma), diluted 1:400 in PBS-10% FES, was added to the plates, and incubated for 30 min at room temperature. In the final step,

substrate ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma) containing 0.03% hydrogen peroxide was added to the plates and incubated for 30 min at room temperature. Absorbance at 405 nm was measured with a microplate reader (Bio-Tek instruments) after 30 min. The amount of IFN- γ in samples was determined by extrapolation to the standard curve.

Con A-induced proliferation. Spleen cells, 2×10^5 cells/well, were cultured in triplicate with either medium alone or varying concentrations of Con A (Calbiochem-Behring Corp., La Jolla, CA), in 96-well, flat bottom tissue culture plates (Falcon), and maintained for 3 days at 37°C in a 5% CO₂-humidified incubator. [³H]-Thymidine (0.5 μ Ci/10 μ l; 5 Ci/mmol; DuPont NEN, Boston, MA) was added for the final 18 h of culture. Cells were harvested onto glass fiber filter paper (Whatman Inc., Clifton, NJ) with a cell harvester (Skatron). The dried filters were placed in scintillation fluid (Biosafe II, RPI, Mount Prospect, IL), and radioactivity determined with a liquid scintillation counter (LKB, Wallac, Finland).

Flow cytometric analysis. Spleen cells were washed in PBS containing 2% BSA and 0.02% azide (flow wash). Fluorescein-conjugated anti-rat IgM (clone G53-238, diluted 1:40; Pharmingen) and phycoerythrin-conjugated anti-NKR-PIA (an NK cell marker, clone 10/78, diluted 1:40; Pharmingen) or fluorescein-conjugated anti-rat CD8 (clone OX-8, diluted 1:40; Pharmingen) and phycoerythrin-conjugated anti-CD4 (clone OX-35, diluted 1:20; Pharmingen) were added to 2×10^6 cells and incubated at 4°C for 30 min. Cells incubated with flow wash alone were included to determine autofluorescence. Following this incubation, cells were washed twice in flow wash, fixed in PBS containing 1% paraformaldehyde, and stored in the dark for no longer than 2 weeks at 4°C prior to analysis. Two-color fluorescence was analyzed with an Elite flow cytometer (Coulter Electronics, Hialeah, FL), equipped with an argon-laser at 15 mW and excitation wavelength of 488 nm.

HPLC-EC. The HPLC-EC procedure has been described in detail before (6, 11, 12). Briefly, NE in the spleen was extracted with alumina prior to analysis by HPLC-EC. Tissues were homogenized in 0.1 M of HClO₄ with 0.25 μ m of 3,4-dihydroxybenzylamine (DHBA) as the internal standard and were centrifuged at 1000g for 5 minutes. The supernatants were used for the aluminum oxide extraction while the pellets were saved for protein assay (Bio-Rad assay kit). For the estimation of monoamines in the MBH, a volume of 200 μ l of HClO₄ containing 0.25 μ m of 3,4-dihydroxybenzylamine (DHBA) was added as the internal standard to the tubes containing MBH. The tissues were sonicated and centrifuged for 2 min at 1000g. The supernatants were stored at -80°C until analyzed for the concentrations of NE, dopamine (DA), serotonin (5-HT), and their metabolites by HPLC-EC and the pellets were stored for the measurement of protein concentrations. At the time of HPLC-EC analysis, samples were loaded onto a Waters 717plus autosampler (Waters, Milford, MA). Splenic NE concentration was expressed in terms of both pmoles/mg protein and pmoles/mg wet weight of the tissue. NE content in the whole spleen was calculated using NE concentration/mg wet weight in the combined hilar and end region of the spleen (12). The neurotransmitter concentrations in the MBH were expressed in terms of pmoles/mg protein.

Statistical analysis. The data were analyzed by ANOVA. Con A-induced proliferation was analyzed using ANOVA with Con A concentration as repeated measures. Parameters that attained significance following ANOVA (P<0.05) were further analyzed by Fisher's least significant difference test.

Results

Mammary tumor size was similar with no significant differences among the three groups at the beginning of the

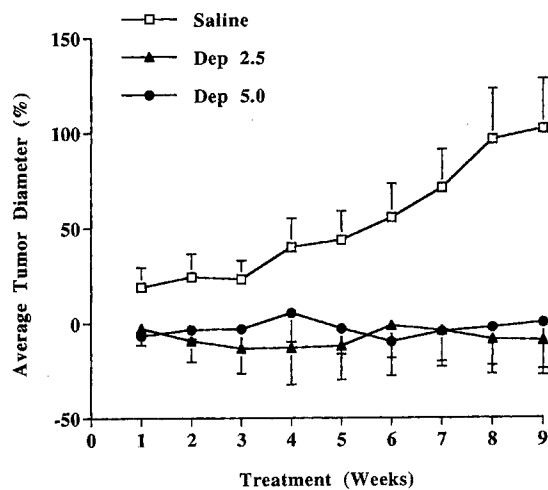


Figure 1. Effects of *i. p.* administration of 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl for 9 weeks on the average tumor diameter in rats with spontaneously developing mammary tumors. Sprague-Dawley female rats (12-mo) were housed in the animal quarters and palpated for the development of tumors every week. After the development of tumors, animals were treated with saline or deprenyl for 9 weeks. *Significantly ($P < 0.05$) different from the Saline group.

Table I. Spleen lymphocyte population from rats with spontaneously developing mammary tumors.

Groups	% sIgM+	% CD4+	% CD8+	% NK+
Saline	40.8±2.4a	39.3±2.4	12.9±0.5	5.3±0.8
Dep 2.5	42.3±1.5	39.9±2.4	17.1±1.5b	4.9±0.5
Dep 5.0	39.9±2.8	41.8±2.3	16.1±1.2b	4.5±0.6

a All values are Mean±SEM

b Significantly ($P < 0.05$) different from Saline

treatment. As shown in Figure 1, tumor diameter increased significantly in the saline-treated group during the 9-week treatment period to more than 100% of the initial size at the end of the treatment period. In contrast to saline-treated rats, there was a significant inhibition of tumor growth in deprenyl-treated rats that was apparent from the fifth week of treatment. Among the 10 rats in the saline group, 7 rats had a consistent increase in tumor diameter and 3 rats showed a slight or no increase in tumor diameter. In contrast, in the 2.5 mg/kg deprenyl-treated group, the tumor diameter decreased in 5 rats, remained unaltered in 4 rats, and increased in 3 rats. Similarly, 6 rats showed a decrease in tumor diameter, 2 rats had no alterations in tumor diameter, and 3 rats had an

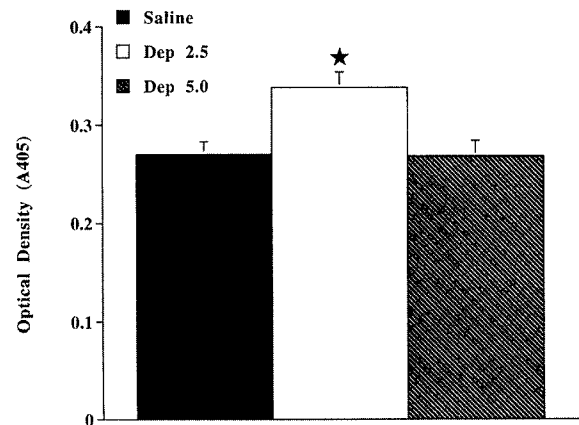


Figure 2. IFN- γ production by spleen cells from rats with spontaneously developing mammary tumors after 9 weeks of treatment with 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl. Spleen cells were incubated with 1.25 μ g/ml of Con A for 24 hrs. Supernatants were tested for IFN- γ by ELISA. *Significantly ($P < 0.05$) different from Saline group.

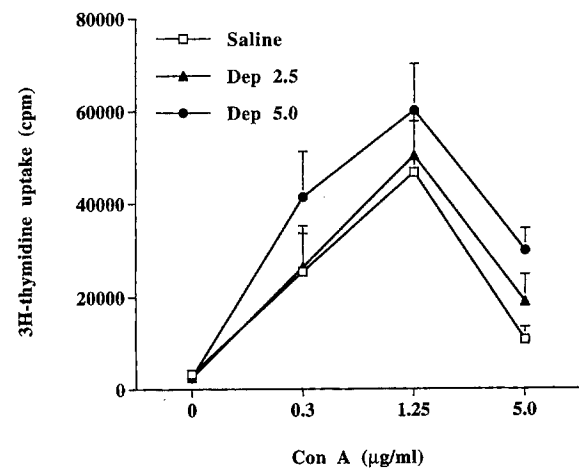


Figure 3. Con A-induced T lymphocyte proliferation by spleen cells from rats with spontaneously developing mammary tumors after 9 weeks of treatment with 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl. Spleen cells were incubated with 0, 0.3, 1.25, or 5 μ g/ml of Con A for 72 hrs. Proliferation of T lymphocytes at 5 μ g/ml of Con A was enhanced in rats that were treated with 5.0 mg/kg deprenyl.

increase in tumor growth in the 5.0 mg/kg deprenyl-treated group.

There were no significant differences in the number of tumors and body weight between the three groups during the 9-week treatment period (data not shown).

IFN- γ was measured in supernatants obtained from Con A-stimulated splenocytes (Figure 2). IFN- γ production was significantly ($P < 0.05$) increased in spleen cells from rats

Table II. Effects of deprenyl treatment on splenic norepinephrine (NE) concentration in rats with spontaneously developing mammary tumors.

Groups	Whole Spleen wt. (g)	NE concentration in Spleen				NE content in Whole spleen (pmoles/mg wet wt.)
		Hilar region		End region		
		pmoles /mg protein	pmoles /mg wet wt.	pmoles /mg protein	pmoles /mg wet wt.	
Saline	0.75±0.11 ^a	9.6±1.9	372.6±79.9	13.4±2.9	485.5±113.1	2245.9±460.7
Dep 2.5	0.72±0.06	15.1±1.6 ^b	552.4±52.7 ^b	14.0±2.7	520.7±69.1	2434.3±294.1
Dep 5.0	0.67±0.04	19.9±3.7 ^b	683.3±115.9 ^b	21.6±3.3	765.3±111.1	3320.8±490.8

a All values are Mean±SEM

b Significantly (P<0.05) different from Saline

treated with 2.5 mg/kg deprenyl. *In vitro* Con A-induced T cell proliferation was unaltered among the three treatment groups at suboptimal and optimal doses of mitogen, but it was significantly (P<0.05) higher in 5.0 mg/kg deprenyl-treated rats at 5.0 µg/ml of Con A (Figure 3). Deprenyl treatment induced no significant modification in the percentage of sIgM+ B cells, CD4+ T cells, and NK+ cells in the spleen (Table I). However, there was a slight increase in the percentage of splenic CD8+ T cells of rats treated with 2.5 mg or 5.0 mg/kg deprenyl.

The concentration of NE (per mg protein and per mg wet weight) was elevated significantly (P<0.05) in the hilar regions of spleens from rats treated with 2.5 mg/kg and 5.0 mg/kg deprenyl in comparison to rats treated with saline (Table II). Spleen weight was unaltered by deprenyl treatment.

The concentrations of NE and serotonin (5-HT) were significantly (P<0.001) higher in the MBH of rats that received 2.5 mg/kg and 5.0 mg/kg deprenyl (Table III). The concentration of 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-HT, was unaltered in the MBH of deprenyl-treated rats. The concentration of the dopamine (DA) metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), was significantly (P<0.001) lower in the MBH of deprenyl-treated rats. Treatment with 5.0 mg/kg increased (P<0.001) DA concentration in the MBH.

Discussion

The results from the present study demonstrate that deprenyl treatment of old female rats with spontaneously occurring mammary tumors arrests tumor growth and simultaneously enhances catecholaminergic activity in the MBH and spleen, and immune reactivity in the spleen. Although the enhancement of neuronal activity in the central and peripheral nervous systems by deprenyl may not be the sole cause for the increment in immune responses, the results nevertheless provide evidence for the inhibition of mammary tumor growth through interactions between the central and peripheral nervous systems and the immune system.

Table III. Effects of deprenyl treatment on catecholamines, indoleamine, and their metabolites in the medial basal hypothalamus (MBH) of rats with spontaneously developing mammary tumors.

Groups	NE	DOPAC	DA	5-HIAA	5-HT
Saline	181.2±13.1a	24.8±3.5	43.5±2.4	68.2±6.9	63.1±2.9
Dep 2.5	261.3±21.3b	14.7±1.4b	50.2±4.5	69.6±3.9	98.7±6.1b
Dep 5.0	306.9±20.9b	8.5±1.8b	67.7±9.2b	58.9±5.3	142.5±12.5c

a All values are Mean±SEM

b Significantly (P< 0.05) different from Saline

c Significantly (P< 0.05) different from Saline and Dep 2.5

Alteration in the hormonal status of the female rat as it proceeds from regular estrous cycles to anestrus stage promotes the development of spontaneous mammary tumors in old rats (1, 7). The three major hormones that determine the development and growth of mammary tumors are prolactin (PRL), estrogen and progesterone, but other hormones including thyroid hormones, growth hormone, insulin and growth factors also can influence mammary tumorigenesis (1, 14). In rodents, persistent hyperprolactinemia significantly increases the incidence of spontaneously developing mammary tumors while hypoprolactinemia reduces the development of these tumors (15). Treatment of old female rats with deprenyl for a period of 8 months reduced the incidence of spontaneously occurring mammary tumors and pituitary tumors in association with a decrease in PRL secretion and monoamine metabolism in the MBH (7). These findings indicate that deprenyl is capable of preventing the age-related increase in PRL levels by augmenting central catecholaminergic activity. Acute administration of deprenyl to young female rats also reduced serum PRL, confirming the inhibitory effects of deprenyl on PRL secretion (16). In the present study, the concentration of DA, the principal inhibitory neurotransmitter of PRL secretion, released from the tuberoinfundibular

dopaminergic (TIDA) system in the MBH, is elevated following deprenyl treatment. An increase in dopaminergic activity in the MBH suggests that deprenyl may suppress PRL secretion through the release of DA into the anterior pituitary (17, 18). Although PRL was not measured in this study, we have previously shown that deprenyl treatment produced a marked reduction in serum PRL and an increase in the concentration of DA in the MBH of young and old tumor-bearing rats (5, 7). Serotonin in conjunction with tumor necrosis factor has been reported to prevent tumor growth by decreasing in blood flow to the tumors and inducing hemorrhagic necrosis (19, 20). It is possible that an increase in the concentration of serotonin in the MBH may exert similar anti-tumor effects on the growth of mammary tumors.

The progressive age-related decline in TIDA activity in the MBH is facilitated by the preovulatory surge in estrogen and PRL secretion during each estrous cycle. This repeated increase in hormonal levels in young rats is neurotoxic to TIDA neurons, resulting in the development of pituitary prolactinomas and hyperprolactinemia in old rats. Long-term ovariectomy and treatment with ergot derivatives have been successful in greatly reducing the incidence of tumors, suggesting that neuroprotection of TIDA neurons may partially aid in preventing the development of tumors (21). Pre- and post-treatment of carcinogen treated rats with deprenyl prevented the development of mammary tumors, possibly through neuroprotection and an enhancement of the tuberoinfundibular dopaminergic (TIDA) neuronal activity in the MBH (6). Several studies support the view that deprenyl is a neuroprotective and neurorestorative agent. For example, deprenyl prevented diminution of tyrosine hydroxylase-positive nerve fibers in the substantia nigra of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, and promoted the regrowth of splenic sympathetic noradrenergic nerve fibers in young sympathectomized and old rats (11, 12, 22).

Cell-mediated immune function is suppressed in tumor-bearing rodents, including reductions in lymphocyte proliferation, delayed-type hypersensitivity, and cytolytic and cytotoxic functions (3). Treatment of tumor-bearing rats with 2.5 mg/kg deprenyl increased splenic IFN- γ production while both doses of deprenyl increased the percentage of CD8+ T cells. The lack of alteration in splenic IFN- γ production in rats that were treated with 5.0 mg/kg deprenyl is not known. Deprenyl may also modulate the production of a number of other cytokines that influence anti-tumor immunity. Administration of deprenyl to rats with carcinogen-induced mammary tumors and to old male rats stimulated splenic IL-2 and IFN- γ production and increased NK cell activity (12, unpublished data). An increase in splenic IFN- γ production may aid in the regression of tumors because of the capability of this cytokine to elicit a potent immune response within the

tumor and to activate NK cells. The moderate increase in CD8+ T cells suggests that an anti-tumor effect of deprenyl may have been achieved, in part, through these effector cells critical to the destruction of tumor cells, but it is yet to be determined whether deprenyl can also enhance anti-tumor cytolytic activity.

An increase in immune responses following deprenyl treatment of tumor-bearing rats may be due to an increase in NE concentration in the spleen. Several lines of evidence indicate that NE modulates immune responses in spleen and lymph nodes. In young mice, destruction of NA nerve terminals by chemical ablation with 6-hydroxydopamine results in depletion of NE in the periphery and diminished T cell-mediated immune responses, including delayed hypersensitivity, cytotoxic T lymphocyte activity, Con A-induced T cell proliferation, and IL-2 and IFN- γ production (13, 23). Treatment of old male rats with deprenyl reversed the age-related decline of NA innervation in the spleen and also improved splenic NK cell activity and IL-2 production (12). Rats with carcinogen-induced mammary tumors had lower levels of splenic NE concentration, and deprenyl treatment restored NE content in the spleen (unpublished data). Collectively, the evidence indicate that immunosuppression may correlate with a decline in splenic NE content.

Alterations in hypothalamic DA and serotonin levels change hormonal secretion and also alter immune responses. Anterior and posterior hypothalamic lesions have resulted in altered splenocyte and thymocyte numbers, decreased Con A-induced T lymphocyte proliferation, NK cell activity, and antibody production, suggesting that these areas in the hypothalamus are involved in the stimulation of cell-mediated and humoral immunity (24). Normal physiological levels of serum PRL in tumor-bearing rats following deprenyl treatment may also elevate splenic IFN- γ production, as PRL is known to enhance NK cell activity, and IL-2 and IFN- γ production (25).

In summary, we have shown that deprenyl can inhibit mammary tumor growth. This reduced tumor growth is accompanied by an increase in hypothalamic DA, NE, and serotonin content, splenic NE concentration, and immune responses in old female rats with spontaneously developing mammary tumors. Deprenyl may inhibit tumor growth by several mechanisms. These results suggest that one of the mechanisms may be through communication between the nervous system and the immune system to enhance anti-tumor immunity in tumor-bearing rats.

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Neural influence on immune responses: underlying suppositions and basic principles of neural-immune signaling

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Introduction

For centuries, observations in the field of medicine have described a powerful influence of the 'mind' on an individual's state of health, susceptibility to illness, and the ability to survive or recover from illness. Until recently, western medicine has paid little attention to such observations; they were considered 'anecdotes' or descriptions of outlying results or events. Western medicine has focused increasingly on a reductionistic, hypothesis-testing scientific model of disease, to understand the mechanisms of action that underlie the present traditional therapeutic approach to medicine. However, recently, more emphasis has been directed towards understanding 'systems biology', such as immunophysiology in the whole organism. In addition, the changing scope of medical treatment towards managed care has brought increasing pressure for integrative or 'whole person care'. The psychological, emotional, and spiritual side of patient care, not previously emphasized in the Western approach to medicine, are re-emerging as important issues. It is a daunting task to under-

take hypothesis-testing, experimental research approaches to elucidate the role of these factors in the cause, course of, and recovery from, disease.

Recent molecular, cellular, and physiological findings have re-focused scientific attention on important functional links between the brain and the immune system (Reichlin, 1993; Ader et al., 1995; Madden and Felten, 1995). Cells of the immune system possess receptors for many neurohormones and neurotransmitters, and show marked alterations in function when these receptors are activated (Sanders and Munson, 1984, 1985; Sanders and Powell-Oliver, 1992). Nerve fibers, using norepinephrine (NE) or neuropeptides as their neurotransmitters, have been found extending into the parenchyma of bone marrow, thymus, spleen, lymph nodes, and mucosal-associated lymphoid tissue. Behavioral or pharmacological manipulation of these neural-immune signaling systems can alter innate immunity, acquired immunity, autoimmunity, inflammatory processes, and immunosenescence. Such altered signaling can change the course of, and response to, infectious diseases, tumors, and other immune-mediated events, and can even alter the efficacy of classical cancer chemotherapy (Madden et al., 1995; Zorzet et al., 1998).

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The field of neural-immune signaling is coming full circle, bringing the hypothesis-testing, experimental scrutiny of molecular and cellular biology to bear on highly complex issues of 'mind-body medicine'. At stake is possible insights into the underlying means by which psychological, emotional, and spiritual factors might alter the course of disease, and how we might use new behavioral and pharmacological approaches to maintain wellness and contribute positively to healing and to recovery from disease.

Underlying suppositions and basic principles of neural-immune interactions

1. *The disease model of western medicine is valid.* Since the substantiation of the microbial basis for infectious disease, western medicine has focused on the mechanistic causal basis for disease. This has led to a somewhat artificial separation of mechanistic factors of disease from contributory factors related to stressors and psychosocial conditions (Gilbert, 1998). Application of insights from neural-immune signaling as a major contributor to mind-body medicine is directed towards bringing the strongest and best physiological states of an individual for the application of classical western medicine (Berk et al., 1989; Zorzet et al., 1998). This should not be viewed as 'alternative medicine', because it is not intended as an alternative to treatment of disease-causing organisms with appropriate antibiotics, or treating cancers with appropriate surgical excision, chemotherapy, or radiation therapy. Rather, it is integrative medicine that seeks to allow current therapeutic approaches to have the best possible effects by optimizing a patient's physiological state.

2. *Susceptibility to disease and recovery from disease depend upon the past and present state of the organism* to resist infections and tumors, to produce a physiological state that is protective, or to mount a response to ongoing challenges that can restore homeostasis or wellness (Classen et al., 1994; Ader, 1995; Sheridan, 1998). Mind-body influences that act through neural-immune signaling are not an either-or proposition juxtaposed with alternative choices. Thus, the cynical view that "stress does not cause ulcers, bacteria do" must be

put in an appropriate integrative context. *H. Pylori* bacteria are indeed the causative organism producing ulcers, but those bacteria will cause ulcers only if the internal milieu in which they reside is favorable to allow them a successful foothold for growth and expression. Psychosocial factors that alter the internal milieu may contribute significantly to the successful establishment of a *H. pylori* infection, or protection from such a potential infection.

3. *Many systems in the body that can enhance wellness and provide resistance to some diseases can be influenced by the brain, and are manipulable in both enhancing and inhibiting directions* (Ader et al., 1990, 1996; Madden and Felten, 1995). A variety of stressors, including physical, psychological, exogenous (e.g. alcohol), and endogenous (e.g. IL-1 β from an inflammatory process or infection) can activate the central nervous system (CNS) resulting in outflow from the hypothalamo-pituitary-adrenal axis (HPA), and autonomic nervous system (ANS), especially the sympathetic division (Glaser et al., 1990; Sheridan et al., 1991; Ader, 1995; Ader et al., 1996; Sternberg, 1997; Sheridan, 1998). Classical behavioral conditioning of immune responses also requires central limbic circuits that activate the HPA axis and the sympathetic nervous system (SNS). Exercise can activate some components of the ANS and the HPA, (Pedersen and Nieman, 1998) while relaxation therapy using video images, music, and positive affirmations can reduce the activity of both the HPA and the SNS (Berk and Bittman, 1997).

4. *Aberrant balance of the signaling systems from the brain to the body, especially the HPA axis and the SNS, can increase susceptibility to disease, can prevent recovery from disease, or may actually causally contribute to disease directly.* Experimental evidence has demonstrated that some stressors may reduce wellness and reduce resistance to disease, such as a viral pulmonary infection (Dobbs et al., 1993) or herpes simplex virus infection (Bonneau et al., 1991). A very large literature on the effects of stressors points towards the HPA axis and SNS as the major outflow

systems from the CNS that lead to altered immune reactivity (Sternberg et al., 1989, 1992; Madden and Felten, 1995; Sternberg, 1997). Several possibilities arise from the acknowledgment that altered activity of the HPA axis and the SNS can mediate an adverse impact on health, especially through their actions on the immune system, although not all of these possibilities have been investigated. Cumulative life stressors and stressors associated with illness may affect the quality of life during cancer, or the course of cancer (e.g. Andersen et al., 1998; Clerici et al., 1998), and the efficacy of chemotherapy for cancer (e.g. Zorzet et al., 1998). Poor doctor-patient interactions and poor social support may induce or contribute to a substandard physiological state, or fail to activate an enhanced physiological state necessary for other therapeutic approaches to be effective. Many interventions for patients facing serious illnesses, such as support groups, programs to provide the patient with an active role in controlling the course of therapy, and interventions such as relaxation therapy, meditation, nutritional support, and stress management, may induce helpful, physiological states conducive to healing, wellness, and prevention or recovery from disease (e.g. Classen et al., 1994).

5. *The channels of communication between the CNS and the body are identifiable, are limited in number, and use chemical mediators as a principal means of communication or signaling* (Felten et al., 1993; Bellinger et al., 1994).

(a) *These mediators fall into several categories:*

(1) *Neurotransmitters* – mediators secreted from nerve terminals that act on their cognate receptors on target cells close to the nerve terminals, initiating a change in ion channels or second messengers.

(2) *Neuromodulators* – mediators secreted from nerve terminals that act mainly to modulate the responsiveness of the adjacent target cell to other neurotransmitters or mediators.

(3) *Neurohormones* – mediators secreted from central neurons, anterior pituitary cells, or target glands in the periphery into the general circulation,

under initial regulatory control by the CNS (Reichlin, 1993). These mediators act via the blood at a distance, and have influence on cells that possess receptors that can 'translate the message'. Releasing factors and inhibitory factors for anterior pituitary hormones are synthesized by central neurons and secreted into a private vascular network directed to the anterior pituitary gland, the hypophyseal portal system. The posterior pituitary hormones, oxytocin and vasopressin, are synthesized by central neurons and secreted into the general circulation. Some cells of the immune system can also synthesize and release neurohormones (e.g. oxytocin, ACTH, CRF) into the local microenvironment as paracrine secretions (Smith and Blalock, 1981; Blalock, 1989).

(4) *Paracrine secretions* – mediators that are synthesized by specific cells (e.g. macrophages, lymphocytes), secreted into the local microenvironment, and act on receptors in or on target cells in the local vicinity of the secreting cell. These mediators are not blood-borne. Some mediators may act as neurotransmitters at one site, and as paracrine secretions from other cells at another site (e.g. VIP, CRF).

(5) *Cytokines* – mediators secreted by cells of the immune system, often acting locally as paracrine secretions (e.g. IL-2) and sometimes acting as a hormone, at a distance (e.g. IL-1 β). Other cells can also synthesize and release cytokines, including vascular endothelial cells, CNS glia, and even neurons themselves (Dinarello and Wolff, 1993; Maier et al., 1994; Watkins et al., 1995).

(6) *Chemokines* – mediators secreted by a variety of cell types that act as chemoattractants to regulate trafficking of inflammatory cells and other immunologically-related cells.

(7) *Growth factors* – mediators secreted by a wide variety of cells, including neural, hormonal, and immunological cell types, that stimulate survival, proliferation, growth, or sprouting of specific target cells.

(8) *Colony-stimulating factors* – mediators secreted by supporting cells that promote the growth and proliferation of specific subsets of stem cells which are destined to differentiate into specific mature cell lines, such as granulocytes or erythrocytes.

(b) *Brain function and activity can direct many of these mediators, especially neurotransmitters, neuromodulators, and neurohormones.* This includes brain responses such as perception of sensory stimuli, emotional reactivity to stimuli or complex influences impinging on the individual, and cognitive processes. Molecular signals, both exogenous (e.g. alcohol) and endogenous (e.g. IL-1 β), can also act through CNS mediators to alter the outflow of neurotransmitters, neuromodulators, and neurohormones, especially NE from the SNS and cortisol from the HPA axis (Watkins et al., 1995).

(c) *CNS circuits and activity that lead to the outflow of these mediators, especially those associated with the HPA axis and the SNS, may be controlled behaviorally with such interventions as relaxation therapy and stress management, meditation, exercise, music therapy, and even counseling and peer support (e.g. Berk and Bittman, 1997).* These mediators (e.g. glucocorticoids and NE) can be measured before, during, and after behavioral interventions, and can be correlated with both upstream events (stage of the intervention that induced the mediators) and downstream events (altered immune responses that result from the mediators).

(d) *Pharmacological interventions (e.g. adrenergic antagonists, glucocorticoid receptor antagonists) can also alter the outflow of these CNS-activated mediators and their downstream effects,* which in turn, can alter the susceptibility to, course of, and recovery from, specific diseases, such as pulmonary viral influenza infection (Sheridan, 1998) or autoimmune disease (Sternberg, 1997).

(e) *A principal supposition that follows from the existence and activity of these mediators, is that all energy, fields, forces, 'healing power within', endogenous capacity to heal or recover, or other 'powers' evoked in systems of healing, whether derived through experimentally-based scientific studies or through non-scientific, non-mechanistic means, are expressed via secretion of these mediators.* Obviously, complex issues such as the will to live, a fighting spirit, a positive attitude, or a sense of hope are exceedingly difficult to study from the

perspective of understanding the interactions of the billions of neurons and glia that are involved in ongoing CNS activity. But the downstream consequences of such complex processes on the neurotransmitters or neurohormonal outflow systems can still be measured over time, and consequences for the immune system, heart, or other target tissue can be assessed. These consequences have a direct influence on the outcome of specific diseases. Ancient systems of healing that used terminology that we now consider being non-scientific, may be stating in more abstract terms the same principles of signaling that we now are working out at the cellular and molecular level.

6. *Mediators that are involved in neural-immune signaling can act in singular or independent fashion, but can also evoke effects that are non-linear or follow an inverted U-shaped dose-response curve (Madden et al., 1995).* In addition, neurotransmitters, acting through their receptors on immunocytes, can act synergistically with other neurotransmitters, with cytokines, or immunologic signals such as activators of the T cell receptor, or with colony-stimulating factors (Beckner and Ferrar, 1988; Carlson et al., 1989). Therefore, a knowledge of the concentration of a neurotransmitter or neuromediator, and the status of its receptor on a given population of target immunocytes is not sufficient to guarantee an understanding of the functional consequence; the presence of other signal molecules may alter the responsiveness of its receptor, or may act in a synergistic or counter-synergistic fashion. In addition, it is also necessary to know the status of the receptor linkage with second messenger effects. For example, β -adrenoceptors are upregulated on T lymphocytes in old rodents and elderly humans, but the G-protein linkage is dysfunctional, and catecholamine activation of these receptors does not generate the same second messenger production of cAMP as occurs in similar cells in young animals (Bellinger et al., 1993). Thus, what appears to be upregulated signaling at first glance, based solely on observations of receptor numbers, actually turns out to be dysfunctional signaling. Collectively, the total pattern of mediators that impinges on a specific immunocyte at a specific point in time at a

specific site can produce tremendous complexity in the integrated patterns of action. These effects need to be worked out singly for each mediator, and then collectively, in concert with other mediators.

7. *The effects of behavioral or pharmacological intervention in neural-immune signaling via these mediators, depends on age, gender, past experience, genetic characteristics, and complex psychosocial factors such as personal support and the perception of control.* As noted above, aged T lymphocytes do not respond to signals such as β -adrenergic agonists in as robust a fashion as their younger counterparts. Female Fischer 344 rats generate more robust proliferative responses than their male counterparts (Ackerman et al., 1991a). Female rodents and humans are more susceptible to some autoimmune disorders than their age-matched male counterparts (Wilder, 1995). Past experience, especially during critical periods of development, may alter the responsiveness of some of the mediator systems; separation of rat pups from their mother prior to weaning can result in a permanently altered HPA axis responsiveness in the pups when they reach adulthood (see Ladd et al., Chapter 7, this volume). Exposure of adult animals to very high concentrations of glucocorticoids may lead to neuronal cell death in the hippocampal formation and may dysregulate the feedback dampening of the HPA axis to glucocorticoid secretion (McEwen, 1998). In humans, perception of control and meaningful social support appear to buffer the immunosuppressive effects of a wide range of stressors (Kiecolt-Glaser and Glaser, 1991). Thus, neural-immune signaling, or brain-to-body signaling, may depend critically on a wide range of host-specific factors that can play a determining role in the final functional outcome of such signaling. One size does not fit all, and so-called 'individual differences' in neural-immune signaling may have a rational and understandable, albeit complex, molecular basis.

8. *Receptors for mediators on or in target cells, and the ability of those cells to respond to mediators that are present in the local milieu, determines the final ability, as a final-common-pathway on a molecular level, of target cells to*

respond individually to brain-to-body signaling. This integrative response can influence the overall capacity in the periphery, collectively and integratively, to respond to disease (e.g. Sheridan, 1998; Sheridan et al., 1994).

9. *All processes of mediators and their receptors on target cells can be investigated by hypothesis-testing experimental approaches.* This includes not only neural-immune interactions and other brain-to-body signaling, but also includes interactions among neurons, although neuron-to-neuron interactions are highly complex and very difficult to investigate at present. Highly complex human behavior and belief systems, such as faith, hope, expectations, a sense of well-being, and the will to live, while difficult at present to investigate or explain at a mechanistic level in the brain, nonetheless can be studied secondarily by exploring the spatial and temporal patterns of brain-to-body mediators they induce, and target cell responses to them. Such data will not 'explain' these complex phenomena, but may shed some light on how they influence functional responses in the periphery, such as the immune system.

A summary of sympathetic neural modulation of acquired immunity, autoimmunity, and immunosenescence

This summary briefly describes work from the laboratories of D. Felten, S. Stevens, Bellinger, Madden, Livnat, ThyagaRajan, and associated colleagues characterizing the potential role of sympathetic nerves and the transmitter norepinephrine to modulate immune responses relevant to disease. Noradrenergic (NA) sympathetic nerve fibers innervate the vasculature and parenchyma of both primary lymphoid organs (bone marrow, thymus) and secondary lymphoid organs (spleen, lymph nodes) (Felten and Felten, 1991). These nerve fibers arborize into specific compartments of lymphoid organs (e.g. periarteriolar lymphatic sheath and marginal sinus/zone of the splenic white pulp, and the medullary cords and cortex/paracortex of lymph nodes) (Ackerman et al., 1987), and end in close proximity (as close as 6 nm) to a variety of target cells of the immune system (Felten

and Olschowka, 1987), including macrophages, T lymphocytes (CD4+ and CD8+), granulocytes, and NK cells. Many subsets of these same cells express selective receptor subtypes for catecholamines and for many neuropeptides, permitting direct signaling (Madden et al., 1995). Many neuropeptide-containing nerve fibers (e.g. substance P, calcitonin G-related peptide, vasoactive intestinal peptide) also arborize in the parenchyma of lymphoid organs, separate from the NA nerve fibers (Felten et al., 1985; Bellinger et al., 1990). In addition, neuropeptide Y is colocalized in most, but not all, of the NA nerve fibers in lymphoid organs (Romano et al., 1991).

In the bone marrow, NE can selectively influence stem cell proliferation, particularly of granulocytes, and can synergize the effects of colony-stimulating factors (Felten et al., 1996). In the thymus, NE may influence thymocyte proliferation and differentiation, although this appears to be present mainly in old animals (Maida et al., 1996; Madden et al., 1997). In the spleen and lymph nodes of non-immune animals, NE nerve fibers influence cellularity and trafficking of lymphocytes, T and B cell responses to mitogens, secretion of some cytokines, and immunoglobulin isotype switching (Madden et al., 1994a). In the spleen and lymph nodes of immune challenged animals, NE appears to have a prominent effect at the initiative phase of an immune response, and enhances cell-mediated responses such as generation of cytotoxic T lymphocyte responses and delayed-type hypersensitivity responses; denervation of NA sympathetic nerves results in diminished cell-mediated responses (Madden et al., 1989, 1994b; Felten et al., 1985; Livnat et al., 1985; Madden and Livnat, 1991). Sympathectomy appears to have no influence on humoral (antibody) responses in Th2 dominant strains of mice (BALB/c), but results in enhanced IgG and IgM responses in Th1 dominant strains of mice (C57BL/6) (Kruszewska et al., 1995). The effects of sympathectomy in mice on immune responses are not altered by the presence of RU486, a glucocorticoid receptor antagonist, suggesting that the sympathectomy-induced changes act through removal of NA nerve fibers, not through altered HPA axis activity (Kruszewska et al., 1998). In lymph nodes of Lewis/N rats, a

strain susceptible to induced autoimmune diseases, (Sternberg, 1997) sympathectomy greatly enhances the severity of inflammation and bone erosion (Felten et al., 1992a; Lorton et al., 1996), while removal of substance P nerves with capsaicin treatment is protective. In the periphery, circulating NE appears to inhibit the activities of mature effector cells (Madden et al., 1995).

In aging Fischer 344 rats, the sympathetic NA innervation of the spleen and lymph nodes selectively diminishes with age, apparently in response to antigen exposure across the life span (Felten et al., 1986; Ackerman et al., 1991b; Bellinger et al., 1987, 1992). We have hypothesized that this selective denervation is the consequence of a process of auto-oxidative metabolic destruction of the NA nerve terminals in these organs, brought about by high turnover of NE, which we believe to be cytokine-driven during immunological reactivity in the local secondary lymphoid organ (Felten et al., 1992b). Loss of NA nerve terminals in experimental animal models results in diminished cell-mediated immune responses, the same diminution is also found as an age-related phenomenon in old animals. Administration of low-dose deprenyl to old rats stimulates the regrowth of NA nerve fibers into the spleen and lymph nodes, and results in enhanced T cell proliferation, IL-2 secretion, and NK cell activity (ThyagaRajan et al., 1998a, 1998b). These findings suggest that several characteristics of immunosenescence, especially diminished T cell responses, can be reversed by provoking regrowth of the NA nerve fibers back into secondary lymphoid organs. Deprenyl also stimulates NK cell activity in rats; when we challenged female Sprague-Dawley rats with 9,10-dimethyl-1,2-benzanthracene (DMBA) to induce mammary tumors, doses of deprenyl of 0.25–2.5 mg/kg/day resulted in diminished tumor incidence and tumor number (ThyagaRajan et al., 1998c).

In summary, sympathetic nerves and their principal neurotransmitter, NE, can influence: (a) basic immune cell functions such as proliferation, differentiation, cytokine production, and cell trafficking; (b) acquired immune responses (cell mediated and humoral); (c) autoimmune reactivity in susceptible strains; (d) some functional parameters of immuno-

senescence; and (e) responses to challenge with some tumors, such as DMBA-induced mammary tumors. We are currently exploring both behavioral and pharmacological manipulation of sympathetic and pharmacological manipulation of sympathetic NA signaling and neuropeptidergic signaling of cells of the immune system for potential immunomodulating effects in disease.

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INHIBITORY EFFECT OF DEPRENYL ON TUMOR GROWTH INVOLVES ENHANCEMENT OF CELL-MEDIATED IMMUNITY IN RATS WITH CARCINOGEN-INDUCED MAMMARY TUMORS. S. ThyagaRajan, K.S. Madden, S.Y. Felten, and D. L. Felten. Department of Neurobiology and Anatomy. University of Rochester School of Medicine and Dentistry, Rochester, NY.

In the preliminary studies conducted in our laboratory, administration of deprenyl, a monoamine oxidase-B (MAO-B) inhibitor, enhanced immunological functions [in vitro interleukin-2 (IL-2) production and NK cell activity], reversed the age-related loss of noradrenergic fibers in the spleens of old male rats, and prevented the development of carcinogen-induced mammary tumors in young female rats possibly by conferring a neuroprotective effect on the tuberoinfundibular neurons in the medial basal hypothalamus. The focus of the present study was to investigate whether treatment of young Sprague-Dawley female rats with deprenyl would inhibit the development and growth of 9, 10-dimethyl-1,2-benzanthracene- (DMBA-) induced mammary tumors by augmenting T-cell functions. Female Sprague-Dawley rats (50- to 55-day old) were administered DMBA orally. After the development of tumors, the rats were assigned to various groups and treated intraperitoneally with saline, 0.25 mg, 2.5 mg, or 5.0 mg of deprenyl/kg BW daily for 13 weeks. At the end of the treatment period, there was a significant reduction in tumor growth and tumor number in rats that received 2.5 mg and 5.0 mg/kg deprenyl. There also was a significant increase in splenic NK-cell activity and Con A-induced proliferation of lymphocytes in rats that received 2.5 mg and 5.0 mg of deprenyl. These results suggest that the administration of deprenyl blocked the development and growth of mammary tumors in part by improving immune reactivity.

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**L-DEPRENYL-INDUCED INHIBITION OF MAMMARY TUMOR GROWTH IS
ASSOCIATED WITH ALTERED NEURAL ACTIVITY AND IMMUNE
FUNCTION IN RATS**

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L-deprenyl, a monoamine oxidase-B inhibitor, prevented the age-related decline in sympathetic noradrenergic (NA) innervation in the spleens of old rats and simultaneously, enhanced splenic natural killer (NK) cell activity and Con A-induced interleukin-2 (IL-2) production. In rats with carcinogen-induced mammary tumors (MT), deprenyl treatment decreased the incidence of tumor and increased the tuberoinfundibular dopaminergic (TIDA) activity in the medial basal hypothalamus (MBH). The aim of the present study was to investigate whether alterations in sympathetic NA activity and cellular immune responses in the spleen, and catecholamines (CA) and indoleamines (IA) in the MBH, accompany deprenyl-induced regression of 9, 10-dimethyl-1,2-benzanthracene (DMBA)-induced and spontaneously developing (SD) MT. In the first experiment, young (5-6 mo) female Sprague-Dawley rats with DMBA-induced MT were treated i.p. with 0, 2.5 mg, or 5.0 mg/kg body weight (BW) of deprenyl daily for 13 weeks. In the second experiment, old (19-20 mo) female Sprague-Dawley rats with SD MT were i.p. administered 0, 2.5 mg, or 5.0 mg/kg BW/day deprenyl for 9 weeks.

In rats with DMBA-induced MT, saline-treated tumor-bearing rats had reduced splenic IL-2 and IFN- γ levels, splenic norepinephrine (NE) concentration and MBH TIDA activity compared to animals without tumors. Deprenyl-induced tumor regression was associated with enhanced splenic IL-2 and IFN- γ production, and NK cell activity compared to saline-treated tumor-bearing rats. In rats with SD MT, deprenyl treatment reduced tumor growth and increased splenic NE level and IFN- γ production. In rats with DMBA-induced and SD MT, deprenyl treatment increased the concentrations of CA and IA in the MBH.

These results demonstrate that deprenyl-induced MT regression is associated with the increased sympathetic NA activity in the spleen, TIDA activity in the MBH, and cell-mediated immunity. These results suggest that tumor growth inhibits functions of both immune and nervous systems, and implies that reversal of the inhibition of catecholaminergic neuronal activities of the central and peripheral nervous systems by deprenyl may enhance anti-tumor immunity.

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Deprenyl: A modulator of neuroendocrine-immune interactions in breast cancer

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Deprenyl, a potent and selective inhibitor of monoamine oxidase-B (MAO-B), has a wide range of pharmacological properties that are beneficial therapeutically in the treatment of human neurodegenerative diseases. Recent studies have demonstrated that deprenyl possesses a neuroprotective function that is not dependent on its MAO-B inhibitory activity. The aim of the present study was to investigate whether alterations in sympathetic noradrenergic (NA) activity and cellular immune responses in the spleen, and catecholamines (CA) and indoleamines (IA) in the medial basal hypothalamus (MBH), accompany deprenyl-induced regression of 9, 10-dimethyl-1,2-benzanthracene (DMBA)-induced and spontaneously developing (SD) mammary tumor (MT). In the first experiment, young (5-6 mo) female Sprague-Dawley rats with DMBA-induced MT were treated i.p. with 0, 2.5 mg, or 5.0 mg/kg body weight (BW) of deprenyl daily for 13 weeks. In the second experiment, old (19-20 mo) female Sprague-Dawley rats with SD MT were i.p. administered 0, 2.5 mg, or 5.0 mg/kg BW/day deprenyl for 9 weeks. In contrast to saline-treated rats with DMBA-induced MT, deprenyl treatment inhibited tumor growth, enhanced splenic IL-2 and IFN- γ production, and NK cell activity. In rats with SD MT, deprenyl treatment reduced tumor growth and increased splenic norepinephrine level and IFN- γ production. In rats with DMBA-induced and SD MT, deprenyl treatment increased the concentrations of CA and IA in the MBH. These results suggest that tumor growth inhibits functions of both immune and nervous systems, and implies that reversal of the inhibition of catecholaminergic neuronal activities of the central and peripheral nervous systems by deprenyl may enhance anti-tumor immunity

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SYMPATHETIC MODULATION OF IMMUNITY IN AGING

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The ability of the immune system to function independently of the nervous system has been countered by a number of studies that have provided evidence for a bidirectional communication between the two systems. One of the pathways that connect the nervous system and the immune system is the neuroendocrine system and the other is the autonomic nervous system (2). Behavioral conditioning, lesions in specific regions of the brain, and external environmental factors, such as stress and psychosocial factors, alter immunological functions, suggesting that the immune system is regulated by neurotransmitters, neuropeptides, and hormones (1, 15). Cytokines and other factors produced by cells of the immune system can cross the blood-brain barrier to induce the release of neurotransmitters and hormones from the brain and pituitary, leading to several central nervous system (CNS)-dependent responses such as sleep, thermogenesis, and anorexia (8, 13, 32).

Our laboratory and others have provided evidence that noradrenergic (NA) and peptidergic nerve fibers are present in various compartments of primary and secondary lymphoid organs and that these nerve fibers can modulate immune functions (4). Receptor-

ligand binding studies have demonstrated the presence of α - and β -adrenergic receptors on T and B lymphocytes and macrophages. Pharmacological and surgical manipulation of NA innervation in bone marrow, thymus, spleen, and lymph nodes results in alteration of immune responses, thus establishing that norepinephrine (NE) released from autonomic sympathetic NA nerve fibers transduces the message through adrenergic receptors on the lymphocytes and macrophages (26). Aging is characterized by an increased incidence of cancer, infectious diseases, and autoimmunity accompanied by a profound decline in immunological and neuroendocrine functions (29, 30). In rodents, there are significant alterations in NA innervation in the thymus, spleen, and lymph nodes with advancing age that may contribute to compromised immunocompetence. In this review, we describe age-dependent changes in the pattern of NA innervation in the thymus and spleen, and discuss the role of agents that can function as neuroimmunomodulators in aged populations with immune dysfunction.

I. NA INNERVATION IN THE THYMUS OF AGING RODENTS

The functional integrity of the thymus is maintained until 2 months of age in rodents followed by a rapid decrease in the process of T cell differentiation associated with thymic involution (20). Fluorescence histochemistry for catecholamines and immunocytochemistry for nerve fibers positive for tyrosine hydroxylase (TH+), the rate-limiting enzyme for the catecholamine biosynthetic pathway, have revealed that NA innervation is sparse in young rodents (3 months) but increases in density in aging animals, paralleling the process of thymic involution. In young rodents, NA innervation of the thymus is predominantly localized in the cortex associated with the subcapsular region and around the vasculature at the corticomedullary junction; very few NA nerve fibers are present in the parenchyma (Fig. 1A). In contrast to young rodents, the density of NA innervation is elevated in the cortex, medulla and in the vasculature associated with alterations in the cytoarchitecture of the thymus (Fig. 1B). In conjunction with the age-related increase in the density of NA innervation, thymic NE concentration is markedly higher in old

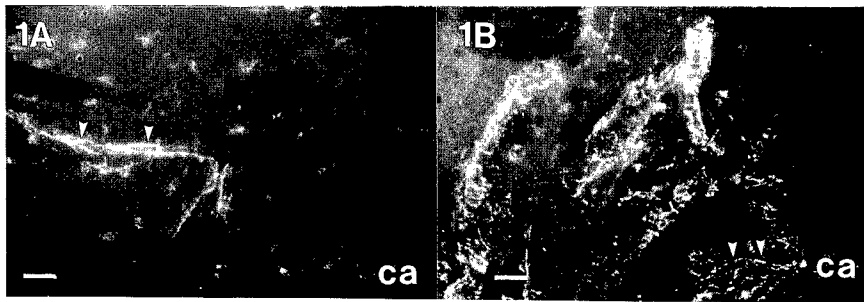


Fig. 1. Fluorescence histochemistry for catecholaminergic nerve fibers in the thymus from young and old BALB/c mice. At 2 months of age, scattered fluorescent nerve fibers are seen in the capsule (arrows, Fig. 1A). By 24 months of age, numerous fluorescent profiles extend from the capsule (ca) toward the center of the thymus (Fig. 1B). Calibration bars = 50 μ m.

rodents but NE content in whole thymus remains unaltered. We believe that the age-associated increase in the density of NA innervation in the thymus is due to a decrease in the weight of the thymus and not due to an increase in the number and amount of NA nerve fibers (5, 27). It is not known, however, whether an increase in NE concentration in the thymus reflects an increase in NE availability leading to increased interactions with thymocytes. Age-related changes in the density of thymic NA innervation are accompanied by reduced thymic cellularity, changes in the proportion of T cell subsets, and infiltration of plasma cells, fibroblasts and macrophages (19, 40, 50). Further studies are required to determine the availability of NE and the nature of adrenoceptor-bearing target cells of the thymus in aged rodents. Currently our laboratory is studying the functional effects of increased NA innervation and NE concentration on differentiation and proliferation of thymocytes in old rodents.

II. NA INNERVATION IN THE SPLEEN OF AGING RODENTS

In young rodents, splenic NA nerve fibers originate from the post-ganglionic cell bodies in the superior mesenteric-celiac ganglion (SCMG) and enter the spleen accompanying the splenic artery with branches extending underneath the capsule (4). Subcapsular plex-

uses follow the trabeculae and vasculature into the spleen, forming dense NA plexuses along the central arteries and extending into the parenchyma of the white pulp. In the white pulp, NA nerve fibers are distributed in the periarteriolar lymphatic sheaths (PALS), mainly among T cells, and in the marginal sinus and marginal zone (Figs. 2A and 3A). Immunocytochemical studies with TH and specific markers for lymphocytes and macrophages have demonstrated that the TH+ nerve fibers are adjacent to T-helper, -cytotoxic, and -suppressor cells within the PALS, are in contact with the macrophages in the marginal sinus, and are in close proximity with the macrophages and B lymphocytes in the marginal zone. A few NA nerve fibers are found among B lymphocytes in the follicles (16, 53). Electron microscopic studies have revealed direct contacts between TH+ nerve endings and lymphocytes or macrophages in the PALS of the splenic white pulp (17).

In F344 rats, NA innervation in the spleen is gradually diminished with age, beginning around 12 months of age (Fig. 3B). Fluorescence histochemistry for catecholamines and double-label immunocytochemistry for TH and immunocytes have revealed a drastic reduction in the density of splenic NA nerve fibers in the PALS, marginal sinus and marginal zone of the white pulp. Reduced NA innervation is associated with loss of T lymphocytes and macro-

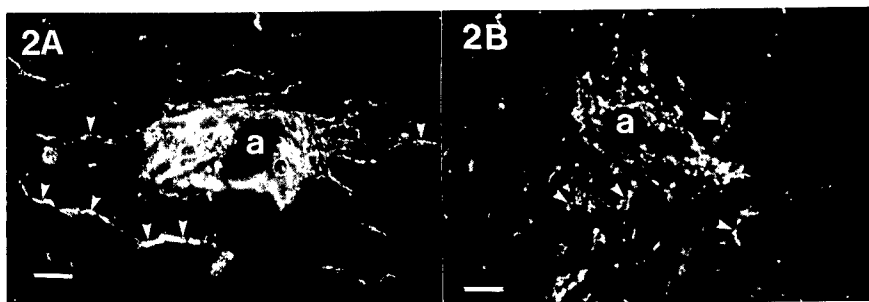


Fig. 2. Fluorescence histochemistry for catecholaminergic nerve fibers in spleens from young and old BALB/c mice. Fluorescent NA nerve fibers are seen along the central arteriole (a) and in the adjoining cellular compartments of the splenic white pulp (small arrowhead) in young 2-month-old mouse (Fig. 2A). By 24 months of age, a slight reduction in NA innervation in the parenchyma around the central arteriole is observed (Fig. 2B). Calibration bars = 50 μ m.

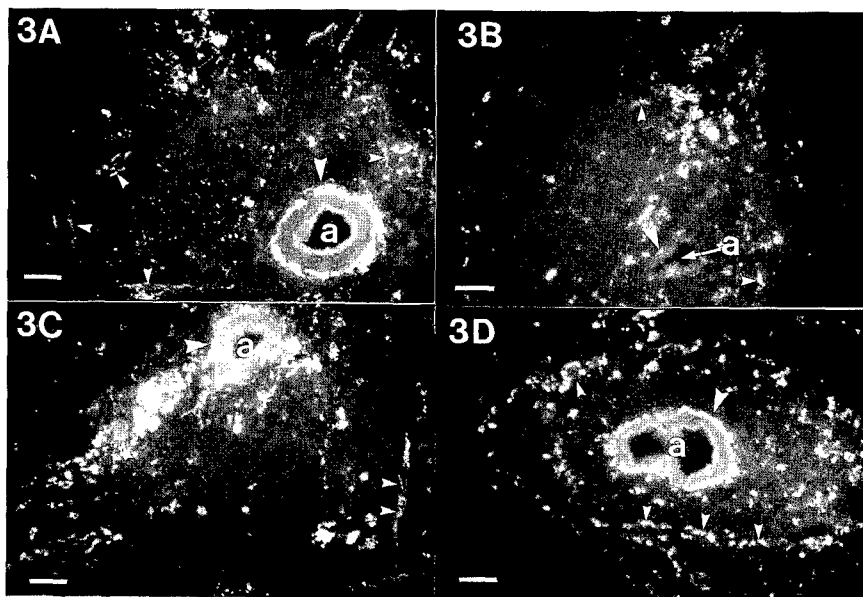


Fig. 3 (A-D). Fluorescence histochemistry in spleens from old F344 rats after 10 weeks of daily deprenyl administration, followed by a 9-day wash-out period. A dense plexus of NA fibers (large arrowhead) surrounds the central arteriole (a) and fluorescent varicosities (small arrowheads) are present in the splenic white pulp of young 3-month-old male F344 rats (A). Sparse innervation of NA fibers is found along the central arteriole (large arrowhead) and in the parenchyma of the white pulp (small arrowheads) in the spleens of untreated (B) 23-month-old male F344 rats. Moderate to intense fluorescent fibers are seen along the central arteriole (large arrowhead) and in the white pulp (small arrowheads) of old rats that received 0.25 mg/kg (C) and 1.0 mg/kg (D) deprenyl. Calibration bars = 50 μ m.

phages in 27-month-old rats. The age-related decline in sympathetic NA innervation in the spleen is accompanied by greater than 50% decrease in NE concentration (6, 7, 18). Neuronal uptake studies with α -methylnorepinephrine in the spleen of old rats have indicated that the loss of NA innervation is due to an actual disappearance of NA fibers, and not because of an inability to detect TH+ nerve fibers in the spleen. We have observed an increase in the density of high-affinity uptake binding sites for NE and an increase in the density of β -adrenoceptors on the splenocytes that together reflect compensatory mechanisms induced following loss of NA innervation

and decreased NE concentration in the spleen (4). The age-related loss in splenic NA innervation may result from alterations in SCMG, including reduced numbers of cell bodies and their reduced ability to synthesize NE (38, 39). Recurrent release of cytokines and other immunological molecules in response to immune challenge may also interfere with NE metabolism in the spleen. Alternatively, the decline in NA nerve fibers may result from a deficiency in the production of target-derived growth factors. In old mice, no alterations in splenic NE concentration have been observed but morphometric analysis revealed a moderate reduction in the density of NA nerve fibers in the spleen (Fig. 2B; 27). Further studies are currently underway in our laboratory to understand the species difference in NA innervation in the spleen and the contribution of age-related changes in NA innervation to immunosenescence.

In young mice, destruction of NA nerve terminals in the splenic white pulp by chemical ablation with 6-hydroxydopamine (6-OHDA) results in diminished T cell-mediated immune responses and enhanced antibody production (23, 28). In addition, adrenergic agonists alter cytotoxic T lymphocyte responses and antibody production *in vitro*, providing additional evidence for a modulatory role of NE in immune reactivity (26). Several of the immunological responses following chemical sympathectomy are similar to those observed in old rodents (4). Specifically, immunological functions, especially cell-mediated immunity, are markedly reduced during aging and following sympathectomy (4, 30). This suggests that changes in immune responsiveness during aging may be associated with diminished NA innervation in the spleen.

III. NEURORESTORATION OF SPLENIC NA INNERVATION IN OLD RATS BY DEPRENYL

L-Deprenyl, a potent irreversible monoamine oxidase-B (MAO-B) inhibitor, is used in the treatment of Parkinson's disease and it improves cognitive performance in patients with Alzheimer's disease (21, 42, 43). Chronic treatment of adult rats with deprenyl increases their sexual activity, and such rats live longer than their untreated peers (22). These beneficial effects may be related to the ability of

L-deprenyl to inhibit dopamine (DA) re-uptake and augment the release of DA from the striatum and to rescue substantia nigra cell bodies from oxidative metabolites as assessed in rodents (12, 27). Recently, several studies have reported that the functions of deprenyl are not limited to its MAO-B inhibitory activity, but may include other actions such as increased synthesis of neurotrophins and enhanced activity of superoxide dismutase and catalase, the principal scavenging enzymes, in the brain (17). In rats, deprenyl treatment protected transected facial motoneurons, and promoted the survival of ganglion cells following damage to optic nerves; such effects may be mediated through an increase in ciliary neurotrophic factor and enhanced cell signaling through receptors for growth factors (3, 9, 14, 47). Altogether, the physiological and pharmacological properties of deprenyl indicate that it has neuroprotective and neurotrophic functions.

We hypothesized that the regrowth of NA nerve fibers in the splenic white pulp after treatment of old rats with L-deprenyl would restore certain cell-mediated immunological functions. In order to determine whether splenic NA innervation could be restored in the aged rat, we treated 21-month-old male F344 rats daily with low doses of L-deprenyl for 10 weeks followed by a 9-day drug wash-out period. Treatment with 0.25 mg or 1 mg/kg deprenyl results in partial regrowth of NA innervation into the splenic white pulp with a corresponding increase in NE content (45). In young rats, splenic NA innervation is lost following destruction of NA nerve fibers with 6-OHDA but NE concentration in spleen is almost restored to normal levels by 56 days (25). Administration of L-deprenyl to 6-OHDA-treated young male F344 rats for 30 days accelerated regrowth of NA nerve fibers into the splenic white pulp and increased splenic NE concentration. Histochemical and immunocytochemical studies have demonstrated the regrowth of TH+ fibers around the central arteriole, in the PALS, and in other compartments of the splenic white pulp in the deprenyl-treated rats (45, 46). In both the young sympathectomized rats and old rats, L-deprenyl treatment does not completely restore NA nerve fibers in the splenic white pulp and the regrowth of nerve fibers occurs mostly in areas close to the hilus and in the middle regions of the spleen. This partial reinnerva-

tion of the spleen by NA nerve fibers is sufficient to increase spleen cell concanavalin A (Con A)-induced interleukin-2 (IL-2) production and natural killer (NK) cell activity in deprenyl-treated old rats (45). L-Deprenyl may act through multiple mechanisms to enhance IL-2 production and NK cell activity. It is unlikely that L-deprenyl acts directly on lymphocytes because *in vitro* addition of deprenyl to splenocytes from young and old rats did not alter Con A-induced proliferation of T lymphocytes and IL-2 production (45).

Nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin-3, neurotrophin-4, and their receptors are expressed in the thymus and spleen (24, 54). In addition, receptors for NGF are present on lymphocytes and monocytes and NGF itself is produced by T and B lymphocytes (35, 37, 44). An age-related decline in the content of NGF has been reported to occur in specific brain regions, but it is unknown whether a similar decrease in NGF activity takes place in the spleen (33, 34). The regeneration and survival of NA nerve fibers in the spleen may also be aided by an increase in superoxide dismutase activity induced by deprenyl. Dietary restriction prevents the decline in superoxide dismutase activity in the spleen and increases IL-2 production by the splenocytes from old rats (10). These findings suggest that the actions of deprenyl on the plasticity of NA nerve fibers in old rats may involve multiple actions at the level of the CNS and the periphery, leading to an improvement in immune reactivity.

IV. DEPRENYL INFLUENCES NEURAL-IMMUNE INTERACTIONS IN MAMMARY TUMORIGENESIS

The pathogenesis of breast cancer is complex and is not attributable to a single factor but to an array of inter-linked factors such as hormonal status, age at first pregnancy, genetic predisposition, diet, and environmental factors (29, 51, 52). These factors influence carcinogenesis through a combination of physical, chemical, genetic, and biological damage to the cells at every stage of tumorigenesis. In chemically-induced and spontaneously occurring mammary tumors in rats, the hypothalamus regulates the development and growth of tumors in association with the anterior pituitary gland and ovaries.

Prolactin (PRL) from the anterior pituitary and estrogen from the ovaries are the two hormones that play a major role in mammary tumorigenesis; they act alone or synergistically to promote the development and growth of mammary tumors. Manipulation of synthesis and release of neurotransmitters and neuropeptides that modulate the secretion of PRL and estrogen regulate mammary tumor growth. A variety of psychological and environmental factors influence the ability of the immune system to react to various stimuli and the magnitude of the response is the ultimate factor in determining the level of carcinogenesis (29, 36).

Treatment of rats with L-deprenyl before and after the administration of the carcinogen, dimethylbenzanthracene (DMBA), inhibits the incidence and development of mammary tumors. These changes in tumorigenesis are associated with inhibition of DA metabolism in the medial basal hypothalamus (47). Administration of L-deprenyl after the development of DMBA-induced mammary tumors inhibits tumor growth and tumor burden associated with enhanced splenic NK cell activity suggesting an immune component to the actions of deprenyl (S. ThyagaRajan, unpublished data). Acute and chronic administration of L-deprenyl inhibits PRL secretion in young and old female rats, and inhibits the incidence of spontaneous mammary and pituitary tumors in old female rats suggesting that the anti-tumor effect of deprenyl is dependent on hypothalamo-pituitary functions (31, 48, 49). We believe that the neuroimmunoendocrine system is involved in the pathogenesis of mammary tumors and breast cancer, and that compounds such as L-deprenyl may be used therapeutically to reverse the disruption of the neuroimmunoendocrine system in breast cancer and prevent mammary tumorigenesis.

SUMMARY

Bidirectional communication between the sympathetic nervous system and the immune system in lymphoid organs is vital for maintaining homeostasis within an organism. It is essential to investigate how the derangement in the signaling between the sympathetic nervous system and the immune system alters immune reactivity. Better understanding of the mechanisms that underlie altered neural-im-

mune interactions in aged individuals is necessary for developing appropriate strategies to combat immunosenescence and diseases whose incidence increases with advancing age.

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