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Effect of Neuropeptide Y on Natural Killer Activity of Normal Human Lymphocytes[1]

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The *in vitro* effect of neuropeptide Y (NPY) on natural killer (NK) cell activities of normal lymphocytes was investigated. NPY at 10^{-9} to 10^{-12} *M* concentrations produced significant suppression of NK activity against K 562 target cells. NPY at 10^{-9} to 10^{-12} *M* concentrations also produced significant inhibitory effects on NK activities of NK-enriched large granular lymphocytes against LAV-infected 8E5/LAV target cells. The suppression was dose dependent against both targets. NPY-induced suppression of NK activity of lymphocytes against K 562 target cells was specifically reversed by rabbit anti-NPY antisera at 1:800 and 1:1600 dilutions, showing the specificity of reactions. Pretreatment of target cells with NPY concentrations capable of inhibiting NK activity did not affect the sensitivity of K 562 target cells for lysis by effector cells. Inhibition of cytotoxicity was not due to direct toxicity of effector cells, because lymphocytes treated with NPY showed normal levels of ^{51}Cr release and their viability was comparable to that of untreated control cells. These studies demonstrated that NPY, a product of sympathetic nervous system activation, may have a significant immunoregulatory effect on NK cell activities of normal lymphocytes that may be of clinical significance.

INTRODUCTION

Recent studies suggest that a bidirectional communication network exists between the central nervous system and the immune system that is primarily mediated through shared neuropeptide signals (Stein, Keller, & Schleifer, 1988; Bateman, Singh, Krol, & Solomon, 1989; Dunn, 1989). Natural killer (NK) cell activity is considered to be an important defense mechanism against virus infections and tumor production and in the regulation of several lymphocyte activities (Herberman & Holden, 1978; Nair & Schwartz, 1981). Stress and depression are known to alter many of the immune responses of the host, including activities, and the number of NK cells, lymphocyte proliferative response to various mitogen and recall antigens, and the production of gamma interferon by lymphocytes (Glaser et al., 1985, 1986; Schleifer, Keller, Camerino, Thorntan, & Stein, 1983; Teshima et al., 1987). Several clinical and preclinical studies have demonstrated that activation of the HPA axis and subsequent alterations in immune responses may not be directly correlated with the physiological changes in circulating corticosteroids levels (Irwin, Daniels, Risch, Bloom, & Weiner, 1988; Kronfol, Hover, Silva, Greden, & Carroll, 1986), although corticosteroids per se are known to inhibit a variety of immune functions *in vitro* (Cupps & Fauci, 1982; Nair &

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Schwartz, 1984, 1988). In a rat model, Shavit and co-workers showed that opioid peptides mediate the suppressive effect of stress on NK activity of splenocytes (Shavit, Lewis, Terman, Gale, & Leibeskind, 1984).

In addition to the central nervous system, an alternative pathway that exerts significant immunomodulatory effect may be the sympathetic nervous system. Noradrenergic nerve fibers form synaptic contacts with lymphocytes and macrophages demonstrating the noradrenergic innervation of lymphoid tissues (Felten, Ackerman, Wiegand, & Felten, 1987; Felten et al., 1988; Felten & Olschowka, 1987; Biondi & Kotzalidis, 1990). Neuropeptide Y (NPY) is a 36-amino acid peptide, neurotransmitter present in peripheral sympathetic nerves that has been hypothesized to regulate immune response during stress. Immunohistochemical and radioimmunoassays showed that NPY is a major neuropeptide in the peripheral nervous system. Previous studies showed that in the periphery, NPY was found colocalized with noradrenaline in a subset of sympathetic neurons (Lundberg et al., 1982; Romano, Felten, Felten, & Olschowka, 1991). Further studies showed that NPY was released during physical exercise (Lundberg, Rudehill, Sollevi, Theodorsson-Norheim, & Hamberger, 1986; Lundberg, Pernow, Fried, & Anggärd, 1987) and potentiated the effect of vasoactive catecholamines (Waeber, 1990; Lundberg et al., 1982), which are known to inhibit cellular immune responses such as NK cell activity (Hellstrand, Hermodsson, & Strannegard, 1985). An interesting observation was that of Reder, Checinski, and Chelmicka-Schorr (1989) who showed that chemical sympathectomy of lymphoid tissues increased NK cell activity. Recently Irwin et al. (1991) showed that chronic activation of the sympathetic nervous system results in the elevation of NPY and is positively associated with a reduction in NK cell activity. This suggests that NPY plays a significant immunoregulatory role in NK cell activity during stress. Although several confounding variables such as stress or catecholaminic release are evidently involved in interaction among the immune, nervous, and endocrine systems, the current study is designed to examine the direct *in vitro* effect of NPY on NK cell activity of normal lymphocytes.

MATERIALS AND METHODS

Blood Donors

Peripheral blood from healthy, adult, male, HIV-seronegative subjects was drawn into a syringe containing heparin (20 units/ml). All donors were apprised of this study and informed consents were obtained consistent with the policies of the National Institute of Health. Subjects were free of medical or psychiatric illness and were not taking medications known to affect immune function, including nonsteroidal anti-inflammatory agents and substance of abuse. Their mean age was 29.24 years (range 20–40 years).

Isolation of Lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood using a modified method of Boyum (1968). Blood was diluted with an equal volume of normal saline and was centrifuged at 400*g* for 30 min at 18°C. The mononuclear cell band was harvested, washed three times with saline, and resuspended in RPMI 1640 medium containing 25 m*M* Hepes buffer supplemented

with 10% heat-inactivated fetal calf serum (GIBCO), 80 μg/ml of gentamicin (Schering, Kenilworth, NJ), and 300 μg/ml of fresh glutamine (complete medium).

PBMC in RPMI 1640 complete medium were depleted of adherent cells by passage through a 7-ml column of Sephadex G-10 beads (Pharmacia Fine Chemicals, Piscataway, NJ). After 45 min of incubation at 37°C, nonadherent peripheral blood lymphocytes were washed through with 1 vol of medium at 37°C. Cell recovery was ~70% of the total input, and monocyte contamination as indicated by nonspecific esterase staining was ~2% (Nair and Schwartz, 1988).

Enrichment of NK Cells

Enrichment of NK effector cells using a discontinuous gradient of percoll (Pharmacia) was carried out as described (Timonen & Saksela, 1980; Nair & Schwartz, 1990). To prepare a discontinuous density gradient, a percoll solution was mixed at various concentrations with RPMI 1640 medium, and 2-ml aliquots (ranges from 50.00 to 37.5% in 2.5% increments) were gently layered into 15 × 130-mm round-bottom, glass test tubes. Lymphocytes depleted of adherent cells were layered on top of the gradient and centrifuged at 300*g* for 45 min at 20°C. Each fraction was collected separately and washed twice in RPMI 1640 medium. The least dense fraction banding at the 37.5% percoll interface consistently showed the highest NK cell activity (Timonen & Saksela, 1980; Nair & Schwartz, 1990) and was used routinely as a source of NK-enriched cells. Approximately 7 to 10% of the input cells were recovered at the interface and 75 to 80% of this fraction were stained with fluorescein isothiocyanate-conjugated monoclonal antibody (1:10 dilution) specific for NKH-1 as analyzed by flow cytometry (Coulter Immunology, Hialeah, FL). Monocyte contamination in purified large granular lymphocyte (LGL) preparation was less than 0.2% as estimated by nonspecific esterase staining as well as CD4 staining by flow cytometry analysis. The remaining cells (20–25%) were CD3 positive.

Tumor Target Cells

The human erythroleukemia cell line, K 562, and a CD4+ CEM-derived subclone of the LAV-infected A3.01 cell line 8E5/LAV were used as targets for NK cells. To 0.8-ml aliquots of complete medium containing 5×10^6 tumor cells, 200 μCi of ^{51}Cr as sodium chromate (New England Nuclear, Boston, MA) was added. The cells were incubated at 37°C for 1 h in a humidified atmosphere of 5% CO_2 in air with intermittent shaking. After incubation, the cells were washed three times with complete medium and resuspended to a concentration of 2×10^5 cell/ml.

Assay for NK Activity

NK activity was determined in a direct ^{51}Cr-release assay as described (Nair & Schwartz, 1990). Briefly, varying concentrations of viable effector cells in complete medium were added to triplicate cultures of ^{51}Cr-labeled target cells in .2-ml volumes in V-bottom microtitration plates (Dynatech Labs, Alexandria, VA). After centrifugation at 40*g* for 2 min, the cells were incubated at 37°C in a humidified atmosphere at 5% CO_2 in air for 4 h. Percentage cytotoxicity was calculated as

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100,$$

where total release represents counts obtained in an aliquot of 1×10^4 target cells, and spontaneous release represents counts released from control wells containing only 1×10^4 target cells. Because the maximum counts (total labeling) determined by either lysing an aliquot of labeled target cells or using unlysed target cells were the same, the latter method was employed to assess total release. Cytotoxicity was also expressed as lytic units (LU)/10^7 effector cells needed to yield 30% cytotoxicity of 1×10^4 target cells. LU were calculated from the cytotoxicity curve by using four different E:T cell ratios for each test by linear regression analysis, as described by Kadish, Tansey, Yu, Doyle, & Bloom (1988).

Human neuropeptide Y was obtained from Sigma Chemical Co. (Cat No. N 5017). NPY was used at concentrations of 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13}, and 10^{-14} *M*. NPY at different concentrations was added directly to the reaction mixture of effector and target cells and incubated at 37°C and NK cell activity was measured using a typical 4 h ^{51}Cr release assay. No preincubation of effector cells with NPY was done.

RESULTS

NPY Suppresses NK Activities of Normal Lymphocytes

Data presented in Fig. 1 show the cytotoxic activity against K 562 target cells of lymphocytes treated with different concentrations of NPY. NPY produced a dose-dependent inhibition of NK activity of lymphocytes; the cytotoxicities in lytic units are 52 ($p < .001$), 54 ($p < .001$), 62 ($p < .005$), 85 ($p < .05$), 108 ($p < .4$), and 112 ($p < .4$), respectively, for 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13}, and 10^{-14} *M* concentrations of NPY compared to 110 LU produced by control lymphocytes. Data presented in Fig. 2 show the effect of NPY on the cytotoxic activity of purified NK cells LGL against HIV-infected 8E5/LAV target cells. LGL treated

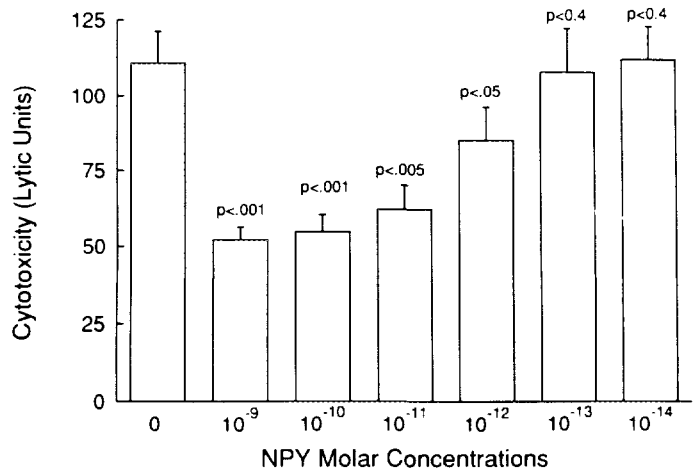

FIG. 1. NPY at different concentrations was added separately to the reaction mixture of effector and target cells and NK activity was measured against K 562 using a 4-h ^{51}Cr-release assay. The cytotoxicity was expressed as lytic units and was calculated from the cytotoxicity curve using four different E:T cell ratios (50:1 to 6.25:1) for each test as described under Materials and Methods. The values are means (±SD) of five separate experiments performed in triplicate using five subjects. The significance of differences between control and NPY-treated cultures was determined by the two-tailed Student *t* test.

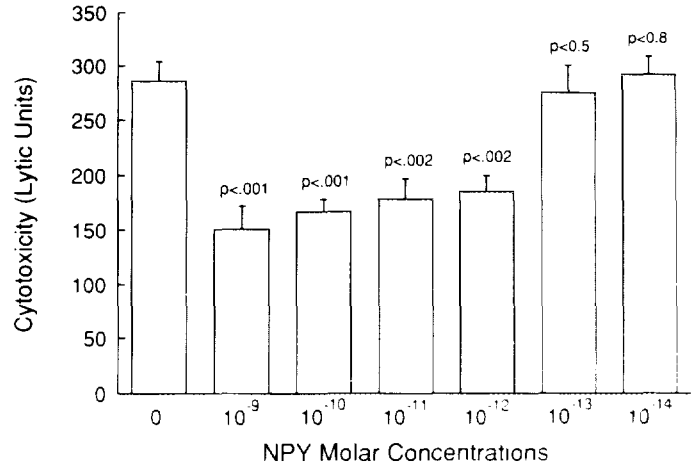

FIG. 2. NPY at different concentrations was added separately to the reaction mixture of effector and target cells and NK activity was measured against LAV-infected 8E5/LAV target cells. The effector cells used were percoll-enriched large granular lymphocytes separated as described under Materials and Methods. The values are means (±SD) of five separate experiments performed in triplicate using five subjects. The significance of differences between control and NPY-treated cultures was determined by the two-tailed Student *t* test.

with different concentrations of NPY produced a dose-dependent suppression of their NK activity. The cytotoxicities are 150 ($p < .001$), 167 ($p < .001$), 179 ($p < .002$), 185 ($p < .002$), 276 ($p < .5$), and 293 LU ($p < .8$) respectively for 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13}, and 10^{-14} *M* concentrations compared to 285 LU produced by control LGL. These studies demonstrate that NPY at concentrations ranging from 10^{-9} to 10^{-12} *M* produced significant suppression of NK activities of lymphocytes as well as NK-enriched large granular lymphocytes.

Reversal of NPY-Induced Inhibition of NK Activity by Anti-NPY Antisera

The effect of anti-NPY antisera on NPY-induced NK inhibition was examined. Data presented in Table 1 show the percentage cytotoxicity of lymphocytes treated with NPY alone, anti-NPY antibody alone, and NPY plus anti-NPY antibody against K 562 target cells. NPY at 10^{-10} *M* concentration produced 25% cytotoxicity ($p < .005$) compared to 48% cytotoxicity produced by control lymphocytes, demonstrating a significant inhibitory effect of NPY on NK cell activity. Lymphocytes treated with anti-NPY antibody at 1:800, 1:1600, and 1:3200 dilutions produced cytotoxicities of 46% (p = NS), 49% (p = NS), and 49% (p = NS), respectively, compared to a similar level of cytotoxicity (48%) produced by control cultures. This suggests that anti-NPY antibody per se did not produce any effect on NK activity of lymphocytes. However, lymphocytes treated with NPY plus anti-NPY antibody at 1:800 and 1:1600 dilutions completely reversed the NPY-induced inhibition of NK activity, percentage cytotoxicities being 46 and 48, respectively, and the cytotoxicities were normalized to that of control lymphocytes (48% cytotoxicity). Anti-NPY antibody at increased dilutions (1:3200) did not reverse the NPY-induced suppression of NK activity; the percentage cytotoxicity (27%) was not significantly different from that of lymphocytes treated with NPY alone (25% cytotoxicity) and the activity was significantly reduced ($p <$

TABLE 1
Reversal of NPY-Induced NK Suppression by Anti-NPY Antisera

Treatment of effector cells	% Cytotoxity
Media	48.7 ± 5.7
NPY (10^{-10} *M*)	25.4 ± 3.4 ($p < .005$)
Anti-NPY antisera (1:800 dil)	46.9 ± 10.6 (NS)
Anti-NPY antisera (1:1600 dil)	49.0 ± 9.0 (NS)
Anti-NPY antisera (1:3200 dil)	49.7 ± 8.7 (NS)
NPY (10^{-10} *M*) + anti-NPY antisera (1:800)	46.7 ± 9.3 (NS)
NPY (10^{-10} *M*) + anti-NPY antisera (1:1600)	48.9 ± 10.7 (NS)
NPY (10^{-10} *M*) + anti-NPY antisera (1:3200)	27.8 ± 3.8 ($p < .005$)

Note. Rabbit anti-NPY antisera (Pennisula Laboratories, Inc., Belmont, CA) at different dilutions were added directly to the reaction mixture of effector and target cells with or without NPY, incubated for 4 h, and NK activity was measured. NK activity is expressed as mean percentage cytoxicity (±SD) of five separate experiments performed in triplicate at a 50:1 E:T cell ratio against K 562 target cells. In another set of two separate experiments, normal rabbit serum was used at similar concentrations as a control for rabbit anti-NPY antisera. The results show that normal rabbit sera did not produce any significant effect on NPY-induced NK suppression (data not presented). The statistical significance of the differences between control and treated cultures was analyzed by a two-tailed Student *t* test. NS, not significant.

.005) compared to that of control lymphocytes. These studies suggest that NPY-induced inhibition of NK activity is specific and can be reversed by anti-NPY antibody.

Effect of NPY on Target Sensitivity to Lysis

To rule out the possibility that NPY-induced inhibition of NK activity of lymphocytes is not due to increased resistance to lysis of target cells by effector cells, targets were preincubated with or without NPY for 4 h at 37°C before use in the cytotoxicity assay. The data presented in Table 2 demonstrate that K 562 target cells, preincubated with 10^{-10} and 10^{-12} *M* concentrations of NPY, showed lysis comparable to that of untreated target cells. These results demonstrate that preincubation of target cells with NPY concentrations capable of inhibiting NK activity did not significantly affect the sensitivity of target cells to lysis by effector cells in the assay. To rule out the possibility that NPY affects the viability of either effector or target cells, effector cells or target cells were preincubated separately with NPY at 10^{-10} and 10^{-13} *M* concentrations and were tested for their viability by trypan blue dye exclusion and the spontaneous ^{51}Cr-release assay. The results showed that NPY did not affect the viability and the spontaneous release of ^{51}Cr

TABLE 2
Effect of NPY on Target Cells

Treatment of K 562	% Cytotoxity
0	46.5 ± 3.2
NPY (10^{-10} *M*)	45.4 ± 4.0
NPY (10^{-12} *M*)	44.2 ± 3.5

Note. ^{51}Cr-labeled K 562 target cells were treated with or without NPY for 4 h at 37°C, washed, and used as target in the NK assay. NK activity was expressed as mean percentage cytotoxicity (±SD) of five separate experiments performed in triplicate at a 50:1 E:T cell ratio.

was negligible (less than 5% of the total labeling; data not presented). These studies demonstrate that NPY-induced suppression of NK cell activity is specific and is not mediated through its effects on susceptibility or viability of target cells.

DISCUSSION

In this study we present evidence that NPY suppresses NK activity of normal lymphocytes *in vitro*. A decrease in the observed cytotoxicity was not due to toxic effects of NPY on effector cells as NPY-treated cells showed levels of viability comparable to untreated control cultures. Also, lymphocytes treated with NPY showed no increase in the spontaneous release of ^{51}Cr compared to untreated lymphocytes. NK cells that were preincubated with NPY for 24 h and then washed to remove NPY also demonstrated significant inhibition of NK activity against K 562 target cells (data not presented). This suggests that the presence of NPY in the effector–target cell mixture is not required to manifest a NPY-induced inhibitory effect. Suppression of cytotoxicity is also not due to the effect of NPY on target cells since target cells treated with NPY for 4 h were as sensitive to lysis by effector cells as untreated control target cells. The suppression was evident at all effector target cell ratios with a wide range of NPY concentrations.

In this investigation, only results of experiments that utilized lymphocytes from normal male donors were presented, since the small number of female samples studied was inadequate for statistical analysis as a separate group. Further, previous studies also showed gender-related differences in various immune responses including NK activity (Ferguson and McDonald, 1985; Hanna & Schneider, 1983; Sulke, Jones, & Wood, 1985; Grossman, 1985; Evans et al., 1992). Therefore, results were presented only from male subjects to avoid confounding variables such as the influences of hormones or the menstrual cycle.

Previous studies indicated a reduced NK cell activity (Anderson, Cardiquist, & Hammond, 1982; Eckstein, Mempel, & Bolte, 1982) and elevated levels of NPY (Maisel et al., 1989) in patients with congestive heart failure. A recent study (Irwin et al., 1991) showed elevated levels of plasma NPY were associated with decreased NK cell activity in patients with depression and Alzheimer care givers, suggesting that NPY plays a significant role in regulation of NK cell activity. The present study provides evidence that NPY can directly inhibit the NK activity of normal lymphocytes.

Since NPY can produce a direct effect on NK cell activity of normal lymphocytes *in vitro*, it is reasonable to suggest that circulating NPY *in vivo* may manifest an immunomodulating effect. The cytotoxicity against 8E5/LAV-infected target cells by LGL may represent NK cell activity since percoll-purified large granular lymphocytes consist mainly of NK cells as demonstrated by NK cell-specific monoclonal marker analysis. Although LGL contain 20–25% T cells, it is unlikely that normal T cells from HIV-negative subjects manifest a classical HIV-specific cytotoxic T lymphocyte reaction during a 4-h period because the T cell cytotoxicity reaction requires at least 4 to 6 days of sensitization to mediate the cytotoxic reaction (Henney, 1977).

The potential mechanisms of the effect of NPY on the NK cell are not clearly understood. It is possible that NPY binds to the NK cell and decreases the cytotoxicity, which is consistent with the earlier observation of innervation of NPY containing nerve fibers to the lymphoid cells (Romano et al., 1991). NPY in synergy with other stress-related hormone may also affect the immune response.

Since NPY is also found colocalized with classical neurotransmitter norepinephrine, it is possible that NPY may exert its immunoregulatory effect by modulating norepinephrine. However, our *in vitro* results cannot support this *in vivo* hypothesis. It is possible that NPY may exert its NK inhibitory effects through different mechanisms. Studies to understand the receptor-specific actions of NPY using NPY antagonists need to be done. The specificity of NPY-induced NK suppression was reversed by anti-NPY antibody, demonstrating the specificity of the reaction. These studies suggest that NPY, a product of sympathetic nervous system activation, may have a significant immunomodulatory effect on NK cell activity, which may be of clinical significance.

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