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Norepinephrine-Mediated Inhibition of Antitumor Cytotoxic T Lymphocyte Generation Involves a β-Adrenergic Receptor Mechanism and Decreased TNF-α Gene Expression[1]

Vladimir V. Kalinichenko,* Margalit B. Mokyr,*† Lloyd H. Graf, Jr.,* Rhonna L. Cohen,* and Donald A. Chambers[2]*†

We have previously shown that norepinephrine (NE) inhibits the in vitro generation of anti-MOPC-315 CTL activity by spleen cells from BALB/c mice rejecting a large MOPC-315 tumor as a consequence of low-dose melphalan (L-phenylalanine mustard (L-PAM)) treatment (L-PAM TuB spleen cells). Since TNF-α plays a key role in the generation of antitumor CTL activity in this system, we determined whether NE mediates this inhibition through inhibition of TNF-α production. Here, we show that NE inhibits the production of TNF-α protein and mRNA by L-PAM TuB spleen cells stimulated in vitro with mitomycin C-treated tumor cells. Flow cytometric analysis of intracellular expression of TNF-α revealed substantial NE-mediated decreases in the percentages of TNF-α^+ cells among $CD4^+$ and $CD8^+$ T cells and $F4/80^+$ activated macrophages. NE inhibition of CTL generation was largely overcome by addition of TNF-α to the stimulation cultures. When the β-adrenergic antagonist propranolol was added to the stimulation cultures of L-PAM TuB spleen cells at a concentration that prevented NE-induced cAMP elevation, the NE-mediated decrease in TNF-α mRNA and NE-mediated inhibition of CTL generation were reversed. Collectively, these results suggest that NE inhibits antitumor CTL generation, at least in part, by inhibiting TNF-α synthesis through a mechanism(s) involving β-adrenergic receptor signaling. ***The Journal of Immunology,*** **1999, 163: 2492–2499.**

Studies of interactions between the nervous system and the immune system are providing insights into the effects of stress on the complex regulation of immune responses (1). Lymphoid organs, including the spleen, thymus, and lymph nodes, are innervated by the sympathetic nervous system, and synapse-like junctions bathed by catecholamine neurotransmitters and other neuromodulators occur between nerve termini and lymphocytes (1–3).

Catecholamines can modulate T cell function both by classical β-adrenergic receptor (β-AR)[3] stimulation (4) and by β-AR-independent mechanisms (5). Catecholamine regulation of T cell function is paralleled by catecholamine regulation of cytokine expression. For example, stimulation of β-ARs by catecholamines, such as norepinephrine (NE) or epinephrine, was reported to be associated with the inhibition of T cell synthesis of TNF-α (6), IL-2 (7, 8), and IFN-γ (7), as well as inhibition of T cell proliferation (8, 9). Catecholamines also act through β-ARs to regulate the expression of cytokine receptors, such as the IL-2 and TNF-α receptors, as well as other surface molecules on immune cells (10–12). We have recently reported that NE acts through β-AR stimulation and the cAMP/protein kinase A (PKA) signal transduction pathway to destabilize the mRNA encoding Thy-1 (a surface glycoprotein implicated in the thymic maturation of T lineage cells (13)); this observation is consistent with possible operation of a general lymphocytic catecholamine/cAMP-responsive mRNA decay system targeting transcripts of genes involved in immunomodulatory effects of stress (14).

Catecholamines exert complex effects on the generation of CTL. For example, while Hatfield et al. (15) and Felten et al. (3) observed stimulatory catecholamine effects on CTL generation against allogeneic tumor cells, we observed a biphasic effect on the generation of CTL activity against syngeneic tumor cells (16). In our studies, NE stimulated CTL generation by BALB/c spleen cells against the syngeneic MOPC-315 plasmacytoma when added at low concentrations, and inhibited the generation of anti-MOPC-315 CTL activity when added at higher concentrations (16).

Since TNF-α was shown to play a key role in the in vitro generation of CTL activity (17), and since catecholamines have been reported to decrease TNF-α synthesis (6, 18), it is of interest to determine whether NE inhibition of antitumor CTL generation occurs as a consequence of an NE-mediated decrease in TNF-α production. As a model in which CTL participate in tumor eradication, we selected the MOPC-315 plasmacytoma. In this system, TNF-α is important not only for the in vitro generation of splenic antitumor CTL activity (17), but also for the in vivo acquisition of potent $CD8^+$ T cell-mediated antitumor cytolytic activity at the tumor site, following low-dose melphalan (L-phenylalanine mustard (L-PAM)) therapy of mice bearing large tumors (19). This CTL activity is responsible for the eradication of the large tumor burden not eradicated by the direct antitumor toxicity of the low-dose L-PAM (20).

Our previous studies defined concentrations of NE and incubation conditions under which NE causes major inhibition of the in vitro generation of anti-MOPC-315 cytotoxicity by spleen cells from MOPC-315 tumor-bearer mice, which have been recently

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[3] Abbreviations used in this paper: β-AR, β-adrenergic receptor; NE, norepinephrine; L-PAM, L-phenylalanine mustard; L-PAM TuB mice, mice treated with a low dose of L-PAM when they bore a large tumor; PKA, protein kinase A.

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treated with L-PAM and cultured in the presence of inactivated tumor cells (16). The current studies were designed to determine whether NE mediates its inhibitory effect for CTL generation by L-PAM TuB spleen cells through inhibition of TNF-α production. Here, we show that NE leads to inhibition of TNF-α production by stimulation cultures of L-PAM TuB spleen cells. Moreover, addition of the β-adrenergic antagonist, propranolol, to these stimulation cultures reversed both the NE-mediated inhibition of TNF-α production and CTL generation.

Materials and Methods

Tumor

The MOPC-315 plasmacytoma was maintained in vivo as previously described (21) as a s.c. tumor in syngeneic 7- to 10-wk-old female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA). Routinely, mice were inoculated with 1×10^6 viable tumor cells, a dose that is ~300-fold higher than the minimal lethal tumor dose, and leads to the appearance of a palpable tumor in 4–5 days. Thereafter, the tumors grow progressively, killing the mice in ~16 days.

Chemotherapy

A fresh stock solution of 10 mg/ml L-PAM (Sigma, St. Louis, MO) was prepared as previously described (22), and was further diluted with PBS (pH 7.2) to the desired concentration just before injection. A dose of 2.5 mg L-PAM/kg body weight was administered i.p. to mice bearing large (~20 mm) MOPC-315 tumors, which resulted from the inoculation of 1×10^6 MOPC-315 tumor cells 10 days earlier (L-PAM TuB mice). This dose of L-PAM is curative for ~90% of the tumor-bearing mice and leads to the complete regression of the s.c. tumor nodule within 8–10 days after L-PAM administration via a $CD8^+$ T cell-dependent mechanism (22, 23).

Spleen cell suspensions

Single cell suspensions were prepared from the spleens of BALB/c tumor bearer mice that had been treated 3–4 days earlier with 2.5 mg/kg L-PAM. Cell suspensions were prepared by mechanical disruption of the spleens between glass slides. In any individual experiment, pooled spleen cells from three to five mice were used.

Reagents and cytokines

NE and propranolol were purchased from Sigma. NE was freshly diluted in DMEM (Life Technologies, Grand Island, NY). Recombinant murine TNF-α was obtained from R&D Systems (Minneapolis, MN).

In vitro stimulation for the generation of CTL

Spleen cells from L-PAM TuB mice were stimulated in vitro with MOPC-315 tumor cells, according to the method we have previously described for the in vitro generation of CTL activity (17). Briefly, 6×10^6 spleen cells were cultured in the presence or absence of 0.2×10^6 mitomycin C-treated (50 μg/ml for 30 min at 37°C) MOPC-315 tumor cells in 24-well plates in the final volume of 2 ml DMEM supplemented with 2 mM glutamine, 50 μM 2-ME, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Sigma), and 5% heat-inactivated FBS (Life Technologies). NE (100 μM) and/or exogenous TNF-α (1 ng/ml) were added at the time of culture initiation. The cultures were incubated at 37°C in 5% CO_2 for 5 days. In some experiments, cultures were incubated with NE (100 μM) for 3 h, and then culture media was replaced with fresh media without NE and the cultures were incubated for 5 days. Propranolol (1 μM) was added to the cultures 15 min before NE.

Antitumor cytotoxicity assay

The cytolytic activity of the cultured spleen cells was determined by the ^{51}Cr-release assay, as previously described (21). Briefly, 1×10^4 ^{51}Cr-labeled tumor cells were incubated with cultured spleen cells at three different E:T ratios in 96-well plates in a total volume of 200 μl of RPMI 1640 supplemented with 10% FBS (Life Technologies). After a 3.5-h incubation at 37°C, 100 μl of supernatant was aspirated and analyzed on an Auto γ Scintillation Counter (Packard Instruments, Downers Grove, IL). The percentage of specific ^{51}Cr release was calculated with the following formula: $[(E^{cpm} - S^{cpm})/(M^{cpm} - S^{cpm})] \times 100\%$, where E^{cpm} represents the ^{51}Cr release by target cells incubated with effector cells, S^{cpm} represents the spontaneous release, and M^{cpm} represents the maximal release obtained by the addition of 100 μl of 2% Nonidet P-40 detergent solution to the target cells. Each experiment was performed at least three times, and the results of a representative experiment are provided as the mean percentages of specific ^{51}Cr release of triplicate samples ± SEM. In addition, to illustrate the reproducibility of our observations, the data from all experiments addressing the same question were converted to lytic units (LU) per 1×10^6 effector cells with the aid of the computer program developed for the Clinical Immunology Service, Program Resources Inc. (Frederick Cancer Research and Development Center/National Cancer Institute, Frederick, MD), where 1 LU is defined as the number of effector cells producing 20% lysis of 1×10^4 target cells. The cumulative data from all experiments are presented as the mean percentage ± SEM of the LU of the control group.

Measurements of IL-2, TNF-α, and IFN-γ concentrations

Spleen cells admixed with mitomycin C-treated MOPC-315 tumor cells were cultured for 24–72 h at 37°C. The supernatants were removed and assayed for IL-2, TNF-α, and IFN-γ using a sandwich ELISA as previously described (24). Rat anti-mouse IL-2 mAb JES6-1A12, rat anti-mouse TNF-α mAb G281-2626, and rat anti-mouse IFN-γ mAb R4-6A2 were used as capture Abs. Rat anti-mouse IL-2 mAb JES6-5H4, rat anti-mouse TNF-α mAb MP6-XT3, and rat anti-mouse IFN-γ mAb XMG1.2 were used as detection Abs. All anti-cytokine mAbs were purchased from PharMingen (San Diego, CA). The sensitivity of the ELISAs was 15–20 pg/ml for all cytokines.

Flow cytometry analysis

Splenocytes admixed with mitomycin C-treated MOPC-315 tumor cells were cultured for 72 h in 24-well plates with 3 μM of monensin (Sigma) present for the last 5 h of the incubation period. Subsequently, the splenocytes were stained with FITC-conjugated anti-mouse CD3, CD4, CD8 (all from PharMingen), or F4/80 (Caltag Laboratories, Burlingame, CA) mAbs. The cells were then fixed, permeabilized by saponin (0.1% in PBS; Sigma), and stained with PE-conjugated rat anti-mouse TNF-α (MP6-XT22) mAbs (PharMingen). PE-conjugated rat IgG1 (PharMingen) was used as a negative control for intracellular cytokine staining. To demonstrate specificity of staining, the binding of PE-labeled anti-TNF-α mAbs was blocked by preincubating cells with 10-fold excess of the unlabeled anti-TNF-α (MP6-XT22) mAbs.

RNase protection assay

Spleen cells were admixed with tumor cells and cultured in 24-well plates for 4 h with or without NE (100 μM). Aliquots of total RNA from the cultures were analyzed for TNF-α and GAPDH mRNAs by RNase protection assay (14) utilizing the mCK-3 probe template set from PharMingen.

Measurement of cAMP concentration

Spleen cells (5×10^6) were suspended in 1 ml PBS supplemented with 5% heat-inactivated FBS. After a 15-min incubation at room temperature, adenylyl cyclase was activated by the addition of NE (100 μM) or forskolin (100 μM). The incubation continued for an additional 15 min at 37°C, and then cAMP was isolated by liquid phase extraction. Ice-cold ethanol was added to the cell suspension (final concentration of 65% v/v ethanol), and the tubes were centrifuged at $2000 \times g$ for 3 min at 4°C. Supernatants were collected, and the precipitate was washed a second time with ice-cold 65% (v/v) ethanol. Supernatants were combined and centrifuged ($2000 \times g$, 15 min, 4°C). The extracts were dried in a vacuum oven at 50°C, then dissolved in 0.05 M sodium acetate buffer (pH 5.8) containing 0.02% BSA. cAMP concentration was measured by an enzyme immunoassay system (Amersham, Arlington Heights, IL; no. RPN 225).

Statistical analysis

The Student's t test was employed to determine the significance of the differences between groups. A p value < 0.05 was considered significant.

Results

Effect of NE on the in vitro generation of antitumor cytotoxicity and cytokine production by stimulation cultures of L-PAM TuB spleen cells

Spleen cells taken from tumor-bearing mice 3 days after treatment with low-dose L-PAM (L-PAM TuB spleen cells) were cultured in vitro with mitomycin C-treated MOPC-315 tumor cells in the presence or absence of NE. After 3 days, the culture supernatants were assayed by ELISA for TNF-α, IL-2, and IFN-γ concentrations. In addition, on day 5 after culture initiation, the L-PAM TuB spleen

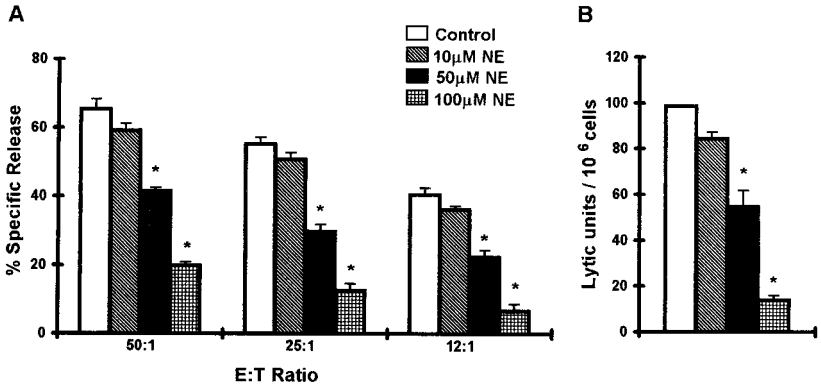

FIGURE 1. Effect of NE on the in vitro generation of anti-MOPC-315 cytotoxicity. Spleen cells taken from tumor-bearing mice 3 days after treatment with a low dose of L-PAM were stimulated in vitro with mitomycin C-treated tumor cells in the absence or presence of NE at the concentrations of 10–100 μM. Anti-MOPC-315 cytotoxicity was assessed on day 5 after culture initiation by ^{51}Cr release assays. *A*, One representative experiment of three yielding similar results is presented as the mean percentage of specific ^{51}Cr release ± SEM. *B*, The cumulative data from all experiments were converted to lytic units (LU) and are presented as percentages of the control ± SEM, where the lytic activity exhibited by L-PAM TuB spleen cells in the absence of NE was considered as 100%. ∗, Statistically significant NE-mediated decrease in the level of anti-MOPC-315 cytotoxicity relative to level exhibited by spleen cells stimulated with MOPC-315 tumor cells without NE ($p < 0.05$).

cells were evaluated for their lytic activity against MOPC-315 tumor cells to confirm the inhibitory activity of NE in this system. As seen in Fig. 1, and in confirmation of our previous observations (16), addition of 10–100 μM NE caused a concentration-dependent inhibition of anti-MOPC-315 cytotoxicity, leading to a 7-fold decrease in $LU_{20}/10^6$ spleen cells at the highest concentration of NE used (100 μM). The overall viability of the spleen cells was not affected by NE at any concentration employed (data not shown). As seen in Fig. 2, addition of 10–100 μM NE also caused a concentration-dependent decrease of TNF-α, IL-2, and IFN-γ levels in conditioned medium collected on day 3 after culture initiation.

Since TNF-α was previously shown to be crucial for the generation of CTL activity by spleen cells from low-dose L-PAM treated mice, and since TNF-α had to be present within the first 48 h of the 5-day stimulation culture to fully realize its potentiation effect for CTL generation in the MOPC-315 system (17), experiments were performed to determine whether NE-mediated inhibition of TNF-α production was evident at earlier times after culture initiation. Time course studies revealed that, within 48 h, the presence of NE (100 μM) was associated with >50% reduction in the concentration of TNF-α in supernatants from stimulation cultures of L-PAM TuB spleen cells (Fig. 3). Similar kinetics for NE-mediated inhibition were observed for IL-2 and IFN-γ production by spleen cells (data not shown).

Effect of NE on intracellular TNF-α cytokine expression by $CD3^+$ and $CD3^-$ populations in stimulation cultures of L-PAM TuB spleen cells

Given the fact that TNF-α can be produced by different cell types, experiments were conducted to determine which cell types produced less TNF-α as a result of exposure to NE. Accordingly, spleen cells from L-PAM TuB mice were cultured with mitomycin C-treated tumor cells in the presence or absence of NE (100 μM). After 3 days, cells were stained with FITC-conjugated anti-CD3 mAbs, then permeabilized and stained with PE-conjugated anti-TNF-α mAbs to demonstrate the presence of intracellular

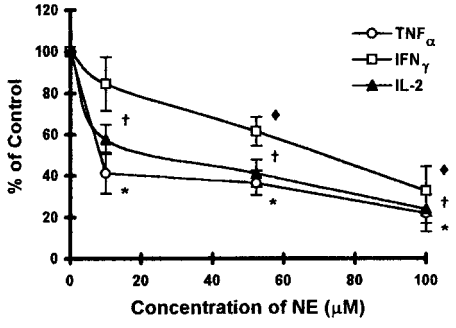

FIGURE 2. Effect of NE on the production of TNF-α, IL-2 and IFNγ. Spleen cells taken from tumor-bearing mice 3 days after treatment with a low dose of L-PAM were stimulated in vitro with mitomycin C-treated tumor cells in the absence or presence of NE at the concentrations 10–100 μM. The concentrations of TNF-α, IL-2 and IFNγ in culture supernatants were determined on day 3 after culture initiation by the use of an ELISA. The cumulative data from three experiments are presented as percentages of the control ± SEM. ∗, †, ♦, Indicate statistically significant decreases in the concentrations of TNF-α (∗), IL-2 (†), and IFN-γ (♦) when compared with relevant cytokine concentration in supernatants of spleen cells stimulated in the absence of NE ($p < 0.05$).

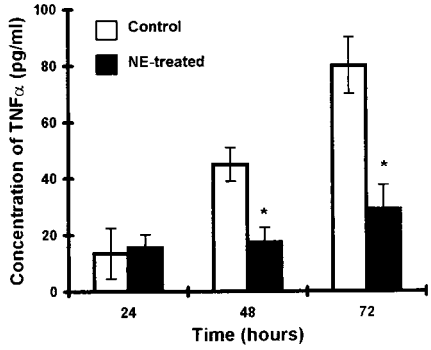

FIGURE 3. Effect of NE on the time course of TNF-α production by stimulated spleen cells. Spleen cells from L-PAM TuB mice were stimulated in vitro with mitomycin C-treated MOPC-315 tumor cells for 24, 48, or 72 h in the presence or absence of NE (100 μM). The concentrations of TNF-α in the culture supernatants were determined by ELISA. The cumulative data from three experiments are presented as percentages of the control ± SEM. ∗, Statistically significant decrease in the concentration of TNF-α in the presence of NE relative to TNF-α concentration in supernatants of spleen cells stimulated in the absence of NE ($p < 0.05$).

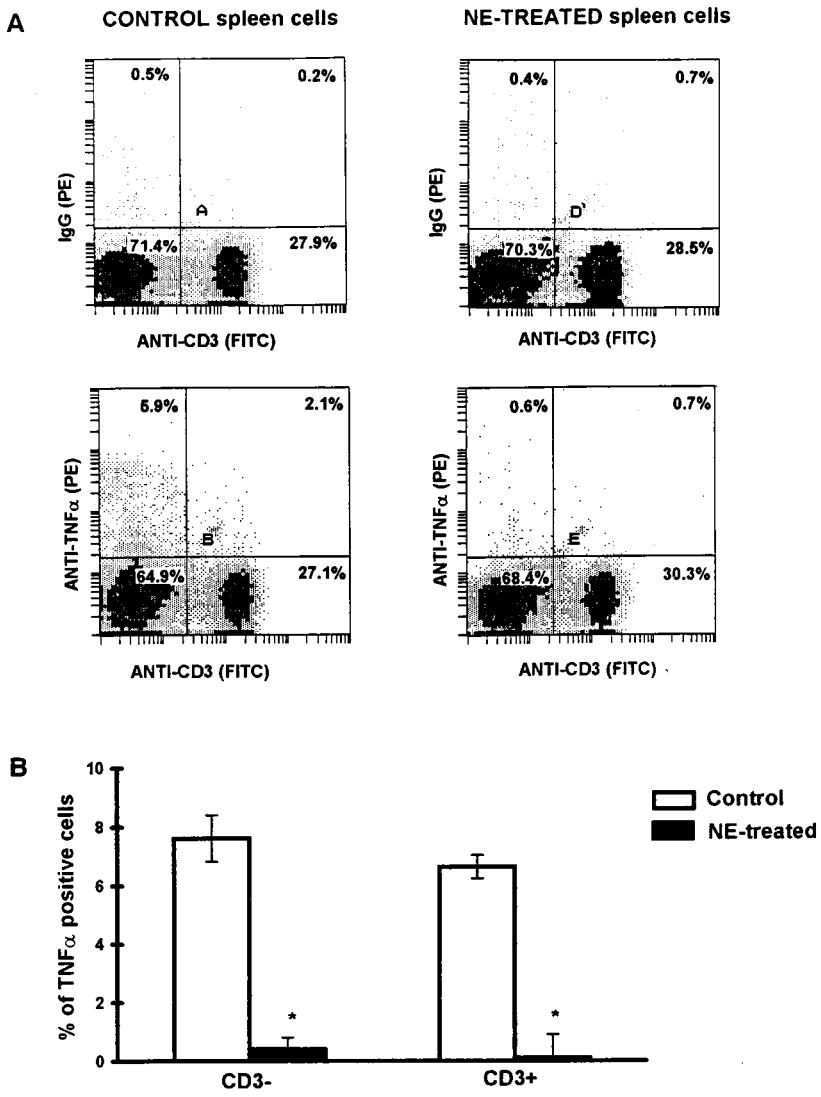

FIGURE 4. Effect of NE on the percentages of TNF-α-positive cells contained within the $CD3^+$ and $CD3^-$ populations of tumor-stimulated splenocytes. L-PAM TuB spleen cells were stimulated with mitomycin C-treated MOPC-315 tumor cells in the presence or absence of NE (100 μM). After 3 days in culture, cells were stained with FITC-coupled anti-CD3 mAb, then permeabilized with saponin, and stained with PE-coupled anti-TNF-α mAb. *A*, Two-color flow cytometric analysis for surface CD3 and intracellular TNF-α expression. Percentages of gated cells are shown in upper right corners of the relevant windows. Three experiments were performed, and the results of a representative experiment are presented. *B*, The percentages of TNF-α-positive cells among $CD3^-$ and $CD3^+$ populations of tumor-stimulated spleen cells. Bars represent percent of $CD3^-$ TNF-α^+ cells among total $CD3^-$ cells and percent of $CD3^+$TNF-α^+ cells contained among $CD3^+$ cells in the absence and the presence of NE. The cumulative data from three experiments are presented as mean $\pm$ SEM. $*$, Statistically significant decrease in the percentages of TNF-α-positive cells in NE-treated cultures relative to that in the control cultures ($p < 0.05$).

cytokine. As seen in Fig. 4*A*, NE treatment did not affect the total percentage of $CD3^+$ cells in the cultures, which was ~30% in the presence or absence of NE. However, a substantial decrease was noted in the percents of TNF-α^+/$CD3^+$ as well as TNF-α^+/$CD3^-$ populations as a consequence of NE addition to the stimulation cultures. This translates into a decrease from 6.6 $\pm$ 0.4 to 0.1 $\pm$ 0.8 in the TNF-α^+ cells among the $CD3^+$ cells ($p < 0.05$) and a decrease from 7.6 $\pm$ 0.8 to 0.4 $\pm$ 0.4 in the TNF-α^+ cells among the $CD3^-$ cells ($p < 0.05$) (Fig. 4*B*). Finally, the NE-mediated decrease in cell-associated TNF-α detected in these assays reflected changes in the levels of cytokine within cells, rather than on their external surfaces, since we did not detect binding of PE-conjugated anti-TNF-α Abs to NE-treated or NE-untreated cells in the absence of permeabilization (data not shown).

Effect of NE on intracellular expression of TNF-α protein by $CD4^+$ T cells, $CD8^+$ T cells, and the F4/80$^+$ subset of splenic macrophages

Since NE reduced the percentage of TNF-α-positive cells among the $CD3^+$ spleen cell population, experiments were performed to determine which subset(s) of T cells was affected by NE. As seen in Fig. 5*A*, NE treatment did not affect the total percentages of $CD8^+$ or $CD4^+$ cells in the cultures. However, addition of NE did lead to a profound decrease in the percentages of TNF-α^+ cells among the $CD8^+$ and the $CD4^+$ cell populations (Fig. 5*B*).

Since NE also reduced the percentage of TNF-α^+ cells among the $CD3^-$ subpopulation of L-PAM TuB spleen cells stimulated in culture with MOPC-315 cells (Fig. 4), and since activated macrophages have also been reported in other systems to be a major source of TNF-α production (24), we assessed whether NE affected intracellular TNF-α expression by cells expressing F4/80$^+$, a specific marker for activated macrophages. As seen in Fig. 5*A*, NE treatment did not affect the total percentage of F4/80$^+$ cells in the stimulation cultures, but addition of NE significantly reduced the percentage of TNF-α^+ cells contained within the F4/80$^+$ cell population (Fig. 5*B*).

Effect of NE on TNF-α mRNA expression by spleen cells

To elucidate the molecular mechanism(s) underlying the NE-mediated inhibition of TNF-α production, we investigated the effect of NE on TNF-α gene expression in L-PAM TuB spleen cells stimulated in vitro with MOPC-315 tumor cells. For this purpose, the spleen cells were admixed with mitomycin C-treated tumor cells and cultured in the presence or absence of NE (100 μM). After 3 h, the cells were harvested, total RNA was prepared, and concentrations of TNF-α mRNA, as well as GAPDH mRNA, were

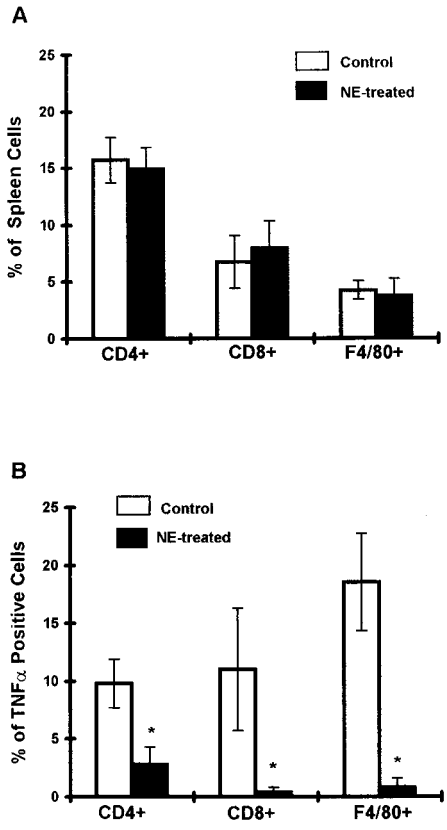

FIGURE 5. Effect of NE on total percentages of $CD4^+$, $CD8^+$, and $F4/80^+$ cells (*A*) and the percentage of TNF-α-positive cells among $CD4^+$, $CD8^+$, and $F4/80^+$ populations of tumor-stimulated splenocytes (*B*). L-PAM TuB spleen cells were stimulated with mitomycin C-treated MOPC-315 tumor cells in the presence or absence of NE (100 μM). After 3 days in culture, cells were stained with FITC-coupled anti-CD4, anti-CD8, or anti-F4/80 mAb, then permeabilized and stained with PE-coupled anti-TNF-α mAb. The percentages of positive cells were determined by flow cytometry. The cumulative data from three experiments are presented as mean ± SEM. ∗, Statistically significant decrease in the percentages of $TNF\text{-}\alpha^+$ cells in NE-treated cultures relative to that in the control cultures ($p < 0.05$).

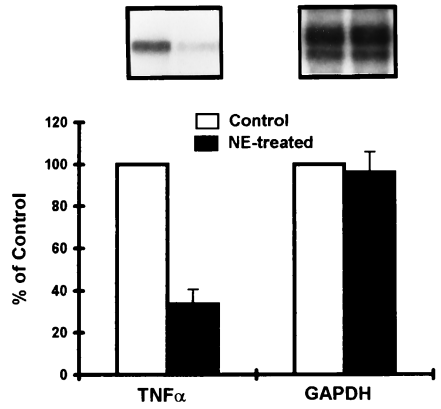

FIGURE 6. Effect of NE on TNF-α mRNA expression by stimulated spleen cells. Spleen cells taken from L-PAM TuB mice were cultured in vitro with mitomycin C-treated MOPC-315 tumor cells in the presence or absence of NE (100 μM). After 3 h, cells were harvested and total RNA was prepared and analyzed by RNase protection assay for TNF-α and GAPDH mRNA expression. Photograph of the results of a representative experiment and cumulative data for three determinations are presented (mean ± SEM).

determined by an RNase protection assay. As part of these studies, we established that no detectable level of TNF-α mRNA was expressed by MOPC-315 tumor cells (data not shown). As shown in Fig. 6, NE caused a dramatic decrease in the level of spleen cell TNF-α mRNA, while not affecting the level of GAPDH mRNA.

Effect of propranolol on NE-mediated inhibition of TNF-α mRNA expression and CTL generation by stimulation cultures of L-PAM TuB spleen cells

Experiments were performed to determine whether signaling through β-ARs is involved in NE-mediated inhibition of TNF-α mRNA expression. For this purpose, L-PAM TuB spleen cells were admixed with tumor cells and incubated in the presence of NE (100 μM) with or without prior addition of propranolol (15 min earlier than NE), at a concentration (1 μM) that antagonized NE-induced cAMP elevation in the L-PAM TuB spleen cells (Fig. 7*A*). Three hours after NE addition, total RNA was prepared and evaluated by the RNase protection assay for TNF-α and GAPDH mRNA levels. Under these culture conditions, propranolol completely overcame the NE inhibitory effect, restoring TNF-α mRNA expression to control levels (Fig. 7*B*).

Since inhibition of TNF-α mRNA expression was evident after 3 h of exposure to NE, and since propranolol completely overcame this inhibition, experiments were performed to determine whether 3 h of exposure to NE also led to inhibition of antitumor CTL generation by L-PAM TuB spleen cells, and, if so, whether propranolol could overcome the NE inhibitory effect for CTL generation. For this purpose, parallel cultures were incubated in the presence of NE with or without propranolol for 3 h, followed by culture for 5 days in the absence of NE or propranolol. As shown in Fig. 7*C*, exposure of L-PAM TuB spleen cells to NE for 3 h led to a substantial inhibition of CTL generation during the subsequent 5-day culture period. This NE-mediated inhibition was largely (40–70%) antagonized by propranolol.

Effect of exogenous TNF-α on NE-mediated inhibition of CTL generation

The close correlation between inhibition of NE-mediated CTL generation and inhibition of TNF-α production suggests that exogenous TNF-α could overcome the NE-mediated inhibition of antitumor CTL generation. To test this hypothesis, L-PAM TuB spleen cells admixed with inactivated tumor cells were exposed to NE (100 μM) in the presence or absence of exogenous recombinant mouse TNF-α at a concentration (1ng/ml) not affecting the generation of antitumor CTL activity in the absence of NE. Fig. 8 shows that exogenous TNF-α substantially offset the inhibitory effect of NE for antitumor CTL generation (50–70%).

Discussion

We have previously shown that catecholamines can suppress the in vitro generation of antitumor cytotoxicity by spleen cells from mice that are in the process of immune-mediated eradication of large MOPC-315 tumors as a consequence of low-dose L-PAM therapy (16). Since TNF-α has been shown to be of crucial importance for CTL generation in this system (17), we hypothesized that the inhibitory effect of NE for anti-MOPC-315 CTL generation is associated with NE-induced suppression of TNF-α production. Here, we show that, indeed, concentrations of NE inhibitory to anti-MOPC-315 CTL generation caused a decrease in the

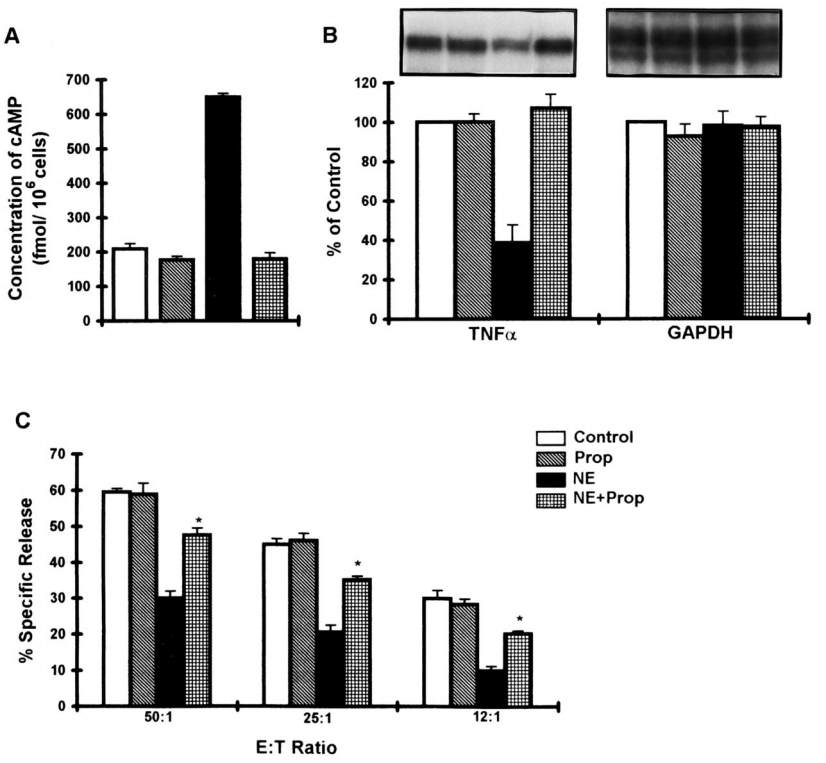

FIGURE 7. Effect of propranolol on NE effect on cAMP concentration (*A*), TNF-α mRNA expression (*B*), and CTL generation (*C*). *A*, Spleen cells from L-PAM TuB mice were treated for 15 min with NE (100 μM) in the presence or absence of propranolol (1 μM), followed by extraction of cAMP, as described in *Materials and Methods*. cAMP concentrations were measured by enzyme immunoassay. One representative experiment of four yielding similar results is presented as means of triplicate determinations ($\pm$SEM). *B*, Spleen cells from L-PAM TuB mice were stimulated with MOPC-315 cells in the presence of NE (100 μM) with or without propranolol (1 μM). After 3 h, total RNA was prepared and analyzed by RNase protection assay. Photograph of the results of a representative experiment and cumulative data for three determinations ($\pm$SEM) are presented. *C*, L-PAM TuB spleen cells were stimulated with tumor cells for 3 h in the presence of NE (100 μM) with or without propranolol (1 μM), followed by centrifugation of cells and replacement of culture medium with fresh medium. Cells were then incubated for 5 days in medium not containing NE or propranolol. One representative experiment of five is presented as the percentages of specific ^{51}Cr release ($\pm$SEM). *, Statistically significant increase in the level of anti-MOPC-315 cytotoxicity in the presence of propranolol and NE relative to level exhibited by spleen cells stimulated with MOPC-315 tumor cells in the presence of NE alone ($p < 0.05$).

concentration of TNF-α in spleen culture supernatants and a decrease in the percentages of TNF-α^+ cells among various splenic subpopulations having potential importance for the generation of a CTL response. Moreover, the addition of TNF-α to the spleen cell cultures largely overcame the NE-mediated inhibition of the generation of the anti-MOPC-315 CTL response, further suggesting that NE down-regulation of TNF-α may be responsible for the decreased antitumor response.

The decrease in TNF-α level upon exposure of the spleen cell cultures to NE was evident at both mRNA and protein levels. The inhibitory effect of NE on TNF-α mRNA levels indicates that mechanisms relating to transcription and/or mRNA stability are involved. However, the possibility that NE also exerts translational or posttranslational effects on the generation or localization of TNF-α protein has not been ruled out by the experiments described in this report.

Our flow cytometric analysis showed that NE caused decreases in intracellular levels of TNF-α protein in at least three spleen cell subpopulations: CD8$^+$ T cells, CD4$^+$ T cells, and F4/80$^+$ activated macrophages. Since all of these cell types can serve as physiologically significant sources of TNF-α, it is reasonable to hypothesize that any or all of them could be key targets for NE inhibition that interrupts a paracrine or an autocrine TNF-α signaling pathway critical for CTL generation. However, despite the

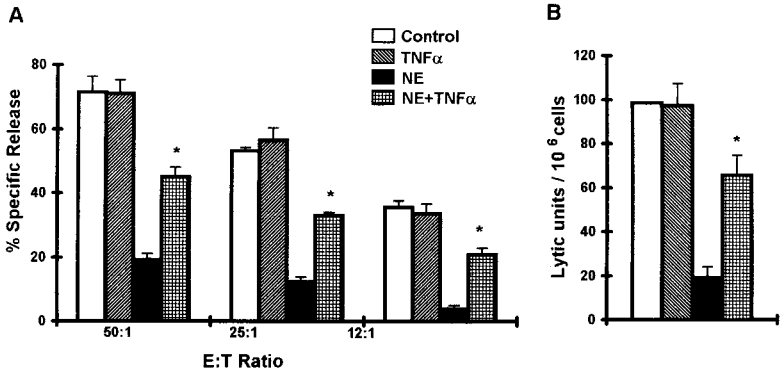

FIGURE 8. Effect of exogenous TNF-α on the NE-mediated inhibition of CTL generation by spleen cells. L-PAM TuB spleen cells were cultured with or without NE (100 μM) in the presence of mitomycin C-treated MOPC-315 tumor cells. TNF-α (1ng/ml) was added to the cultures at the time of culture initiation. The level of anti-MOPC-315 cytotoxicity was assessed by ^{51}Cr release assay on day 5 after culture initiation. *A*, One representative experiment of three yielding similar results is presented as the percentages of specific ^{51}Cr release ($\pm$SEM). *B*, The cumulative data from three experiments were converted to lytic units (LU) and are presented as percentages of the control ($\pm$SEM) where the lytic activity exhibited by L-PAM TuB spleen cells in the absence of NE was considered as 100%. *, Statistically significant TNF-α-mediated enhancement in the level of anti-MOPC-315 cytotoxicity relative to that exhibited by spleen cells stimulated with MOPC-315 tumor cells in the presence of NE ($p < 0.05$).

importance of $CD4^+$ T cell-mediated "help" to many cellular immune responses, our previous finding that depletion of $CD4^+$ T cells from spleen cell preparations just before initiation of stimulation cultures does not affect anti-MOPC-315 CTL generation (25), suggests that the production of TNF-α by $CD4^+$ cells may not be essential for the generation of anti-MOPC-315 cytotoxicity. Thus, the NE-mediated decreases in expression of intracellular TNF-α by $CD8^+$ T cells and/or by $F4/80^+$ activated macrophages are considered to be more likely to have a role in CTL down-regulation in the MOPC-315 system.

We show here that the NE inhibitory effect for CTL generation can be substantially overcome by the addition of exogenous TNF-α. Our finding that TNF-α does not completely overcome the inhibitory effect of NE for CTL generation may be due to the fact that NE also decreases production by the tumor-stimulated spleen cell cultures, of two additional "type 1" cytokines (IL-2 and IFN-γ) that have been reported to promote cell-mediated immunity in a number of systems (4, 26, 27). While the experiments presented in this communication provide support for the idea that TNF-α is important for the generation of antitumor spleen cell CTL activity, possible roles for the observed NE inhibitory effects on IL-2 and IFN-γ also merit consideration. Indeed, results of a preliminary study (V. Kalinichenko, unpublished observations) indicate that addition of exogenous IL-2 also partially restores the generation of antitumor CTL activity in L-PAM TuB spleen cell cultures in the presence of NE, and addition of TNF-α and IL-2 together totally overcame NE inhibition of CTL generation.

In view of previous results suggesting a possible negative role for IFN-γ in the generation of antitumor CTL activity in the L-PAM TuB spleen culture system (19), it does not seem likely that the NE inhibition of IFN-γ production observed in the current studies plays a major role in the inhibitory activity of NE for CTL generation. However, IFN-γ does enhance many cellular immune responses (27, 28). Thus, NE-mediated down-regulation of IFN-γ expression, such as that described here, is likely to have an important role in stress-associated inhibition of host immunity against a variety of infectious agents, parasites, and tumors.

The β-AR antagonist propranolol was found, in our studies, to completely reverse NE-mediated decreases in the amount of TNF-α mRNA in total spleen cell RNAs. In previous studies of cytolytic activity, propranolol was unable to antagonize the inhibitory effects of long-term (5-day) incubation with NE on the generation of antitumor CTL activity (16). In contrast, in the current studies, NE potently inhibited antitumor CTL generation when the spleen cells were exposed transiently (3 h) to NE, and then cultured for 5 days in its absence, and this inhibition was largely antagonized by propranolol (18). These findings indicate that NE-mediated activation of the β-AR/cAMP/PKA pathway can be an effective mechanism for the NE-mediated inhibition of antitumor CTL generation. The potent CTL-inhibitory effects of cAMP/PKA stimulation is further supported by earlier results in our laboratory, wherein cholera toxin, a stimulator of endogenous cAMP production, and dibutyryl-cAMP, a membrane-penetrating analogue of cAMP, also potently inhibited the generation of anti-MOPC-315 cytotoxicity (16). However, the current findings that the propranolol reversal of short-term NE-mediated inhibition of antitumor CTL generation is only partial (40–70%) is consistent with our earlier findings that NE is capable of directing major β-AR-independent inhibitory effects on the generation of tumor-directed CTL immunity (16). Taken together, these findings suggest that the β-AR-independent actions of NE cause only partial, albeit significant, inhibition of antitumor CTL generation if NE exposure is of short duration, but that the β-AR-independent inhibition becomes increasingly prominent as time of exposure of the L-PAM TuB spleen cell cultures to NE is increased. Within 5 days of NE exposure, the β-AR-independent (propranolol-insensitive) component of NE inhibition of CTL generation becomes dominant and obscures the β-AR-dependent (propranolol-sensitive) component.

The ability of NE to suppress the generation of anti-MOPC-315 cytotoxicity by L-PAM TuB spleen cells by down-regulating TNF-α production suggests that stress-induced elevation of NE levels may also down-regulate the in vivo acquisition of CTL activity against tumor cells by down-regulating TNF-α production. Our current studies illustrate that the inhibitory effect of NE upon CTL generation can be largely overcome by addition of exogenous TNF-α. These results suggest that elevation of TNF-α levels in vivo could help to overcome the inhibitory effect of stress-induced NE elevation for the development of CTL activity. Enhanced understanding of these mechanisms may facilitate the therapeutic effectiveness of modalities that depend on the development of antitumor CTL activity for tumor eradication.

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