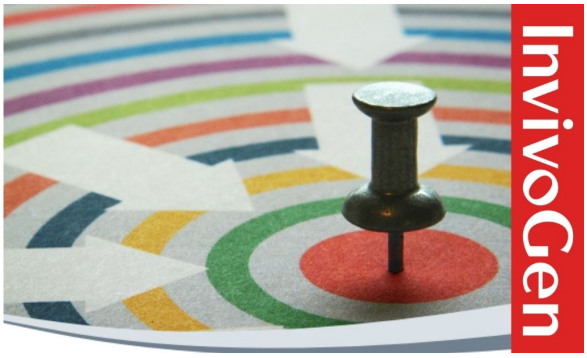

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Endogenous Norepinephrine Regulates Tumor Necrosis Factor-α Production from Macrophages In Vitro[1]

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Evidence for the extraneuronal accumulation of norepinephrine has been demonstrated to occur in macrophage (Mϕ), yet the physiologic role of this system remains undefined. We have assessed the response of murine peritoneal Mϕ to adrenergic antagonists. We have also defined a physiologic role of a Mϕ-associated pool of the nonspecific adrenergic agonist norepinephrine. We investigated the constitutive involvement of α-adrenergic and β-adrenergic receptors in LPS-induced TNF-α production. CFA-elicited Mϕs were incubated with LPS (1 μg/ml) in the presence or absence of adrenergic agonists and/or antagonists. Although stimulation of α-adrenergic receptors increased TNF production and gene expression, β-adrenergic receptors decreased it. Interestingly, when adrenergic antagonists along with LPS alone were added to Mϕ, they generated the response opposite to that produced by their suitable agonist, suggesting a role for endogenous norepinephrine in Mϕ. Thus, although α2-adrenergic antagonists attenuated TNF production, β-adrenergic antagonists augmented TNF expression in a concentration-dependent manner. Norepinephrine and epinephrine were found in Mϕ as determined by HPLC and LPS stimulation induced a significant decrease in their content. Mϕs were also incubated with LPS or medium only, washed, and then challenged 12 h later with LPS. When given a second LPS stimulation, Mϕs were found to have an increased response to α2-adrenergic agonists and decreased response to α2-adrenergic antagonists. Therefore, Mϕ-associated norepinephrine appears to regulate LPS-induced TNF production in an autocrine fashion. *Journal of Immunology,* 1994, 152: 3024.

Mϕs[3] participate in many cellular responses via the production of specific polypeptide mediators, collectively called cytokines. These Mϕ products serve as important cell-to-cell communication signals. Currently, the mechanisms by which these cytokines are regulated are still poorly understood. Because the Mϕ-derived cytokine, TNF, appears to be an important element in many biologic processes, it is critical to understand the endogenous mechanisms that regulate TNF production by Mϕs.

Neurotransmitters, such as the adrenergic mediator norepinephrine, are released from nerve terminals and mediate a large spectrum of biologic responses. Once released, norepinephrine can stimulate either β-adrenergic or α-adrenergic receptors on effector cells. Not only are serum levels of the endogenous adrenergic mediators norepinephrine and epinephrine increased in stressful situations, including various inflammatory responses (1–3), but numerous studies document that physical, chemical, or psychosocial stress can influence immunologic functions (4, 5). There are a number of studies that have examined the changes in immune/inflammatory responses attributed to adrenergic receptor-activating substances (6–10). Overall, these studies have found both enhancement and suppression of responses as a result of selective stimulation of certain adrenergic receptor populations. Norepinephrine will preferentially stimulate one of the two classes of adrenergic receptors in a concentration-dependent manner.

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[3] Abbreviations used in this paper: Mϕ, macrophage; PGE_2, prostaglandin E_2.

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Therefore, depending on the concentration of norepinephrine, the agent may have either immunoenhancing or immunosuppressive effects. Furthermore, it is generally accepted that β-adrenergic and α-adrenergic receptors have different if not opposite functions on effector cells (11, 12). This allows norepinephrine to have diverse activities, depending on the cellular target and the concentration available at the cellular, tissue, or systemic level.

Stress-related neuroendocrine hormones, such as norepinephrine, are involved in the control of phagocyte effector functions (13). Mϕs are one of the cell populations that norepinephrine can stimulate, thus demonstrating a modulatory link between the nervous system and the immune system (16, 17). The β-adrenergic binding site has been identified on specific Mϕ populations (14) and functions as a receptor. On activation of the β-adrenergic receptor, an inhibitory effect on immunologic processes has been documented (15). Recently, it has been demonstrated that stimulation of β-adrenergic receptors on Mϕ regulates the production of Mϕ-derived TNF production (16). The α-adrenergic receptor, which functions in an opposite manner to the β-adrenergic receptor on Mϕ, has also been identified on Mϕ (17). Therefore, different classes of adrenergic compounds can regulate macrophage responsiveness (18–20). Compounds that act via β-adrenergic receptors reduce the activity of immunocompetent cells, whereas compounds that stimulate α-adrenergic receptors augment the responsiveness of immunocompetent cells.

In addition to being key effector cells during an inflammatory response, it is now accepted that Mϕs perform roles other than just the sequestration and destruction of foreign agents. These roles include the release of endogenous pools of hormone/mediators that may direct inflammatory responses and act as mediators of cellular homeostasis. Specific endogenous mediators may act in an autocrine fashion and regulate TNF expression, as has demonstrated for the lipid mediator PGE_2 (21, 22). Mϕs have also been demonstrated to accumulate extraneuronal norepinephrine. Furthermore, the milieu in which the Mϕ resides will dictate the amount of norepinephrine the Mϕ can accumulate and store (23). However, the relevance of this property has not been made clear. Norepinephrine release on LPS stimulation could act in an autocrine fashion and regulate Mϕ responsiveness.

This investigation was undertaken to determine whether endogenous norepinephrine possesses an autoregulatory role on the production of Mϕ-derived TNF. We present data demonstrating that Mϕ possess an endogenous pool of norepinephrine and, on stimulation of Mϕ with LPS, this endogenous pool of norepinephrine regulates TNF expression in an autocrine fashion. Interestingly, the effect of norepinephrine on LPS-dependent TNF expression appears to involve both α- and β-adrenergic receptors that are intimately involved in the regulatory event.

Materials and Methods

Murine Mϕ cultures

Female, CBA/J specific pathogen-free mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments. Mice were housed in pathogen-free quarters and given food and water ad libitum. Elicited murine Mϕs were recruited via an i.p. injection of 0.5 ml CFA (diluted 1:1 with sterile saline). After 14 days the activated peritoneal Mϕs (more than 80% Ia^+) were harvested, washed extensively, and then resuspended in sterile, serum-free RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with: 1 mM glutamine, 25 mM HEPES, 100 U penicillin, and 100 μg streptomycin/ml. Mϕs (1×10^6/ml) were added to either 35-mm (1 ml) or 100-mm (10 ml) culture dishes or 8-well chamber slides (0.25 ml) and incubated at 37°C in 5% CO_2/95% humidified air. After 2 h adherence, the cells were washed three times, and the Mϕ monolayers were overlaid with media containing the appropriate agents described below.

TNF analysis

Cell-free supernatants from Mϕ monolayers were assayed for TNF activity at 30 min after LPS administration (when TNF levels were low) by means of WEHI 164 subclone 13 cytotoxicity assay (24), and at 4 h after LPS administration the higher levels of TNF activity were assessed by using the LM fibroblast cell line cytolytic assay (25). Adrenergic drugs did not affect the TNF response in either of these assays. WEHI cells (5×10^5/1 ml) were cultured in 0.1 ml in 96-well microtiter plates (Costar, Cambridge, MA) with serial dilutions (1:2) of test samples and 0.5 μg/well actinomycin D (Calbiochem, Boehring Diagnostics, La Jolla, CA). Serially diluted recombinant human TNF (Genzyme Corp., Cambridge, MA) was used as standard. After 20 h incubation at 37°C, 20 μl MTT (5 mg/ml; Sigma, St. Louis, MO) was added to the samples and incubation at 37°C was continued for 4 more h. After the incubation, crystals were dissolved by removing 150 μl of the supernatant and 100 μl of a 0.04 N HCl/isopropanol solution was added to all wells and stored in darkness overnight. LM cells (5×10^4/0.1 ml) were cultured in 96-well microtiter plates with serial dilutions (1:2) of test samples and 5 μg/well actinomycin D. After 18 h incubation at 37°C, the plates were washed and the remaining viable cells stained with crystal violet (0.5% in methanol/water 1:4). The level of cell lysis was determined using a MicroELISA Autoreader (550 nm for the WEHI assay and 620 nm for the LM assay). Units of activity were calculated using internal recombinant TNF standards.

Mϕ TNF mRNA analysis

Total Mϕ RNA was isolated according to a modification of the procedures established by Chirgwin et al. (26) and Jonas et al. (27). The Mϕ monolayer from a 100-mm plate (10^7 total Mϕs) was solubilized in a solution of 25 mM Tris, pH 8.0, containing 4.2 M guanidium isothiocyanate, 0.1 M 2-ME, and 0.5% Sarkosyl. After homogenization, an equal volume of 100 mM Tris, pH 8.0, containing 10 mM EDTA and 1% SDS was added, and the entire solution was extracted with chloroform-phenol and chloroform-isoamyl alcohol. The extracted RNA was precipitated in alcohol and the pellet dissolved in 10 mM Tris/0.1 mM EDTA buffer with 0.1% Sarkosyl. Separation of RNA was performed using a formaldehyde/1% agarose gel, and the gel was transblotted to nitrocellulose, baked, prehybridized, and hybridized with a ^{32}P end-labeled oligonucleotide probe. The 30-mer oligonucleotide probe (5′-GTC-CCC-CTT-CTC-CAG-CTG-GAA-GAC-TCC-TCC-3′) was labeled to high sp. act. with T4 polynucleotide kinase and [^{32}P]ATP and used to probe baked filters for murine TNF (27). The filters were exposed to X-OMAT R film (Eastman Kodak Co., Rochester, NY) at −70°C with an intensifying screen. The autoradiographs were quantitated by laser densitometer scan. The total RNA levels per lane on the gel were assessed by monitoring 28S and 18S rRNA.

Immunolocalization

Mϕ cultures in 8-well chamber slides (Lab Tek, Nunc, Inc., Naperville, IL) were fixed for 5 min in 4% paraformaldehyde in PBS, rinsed twice with PBS, then fixed 10 min in a 1:1 mixture of methanol with 3% hydrogen peroxide solution to inactivate any remaining peroxide activity.

A 1:50 dilution of blocking serum was next applied for 10 min at 37°C. The slides were then exposed to optimal dilutions of specific Ab and similar dilutions of control sera. After 10 min incubation at 37°C, the slides were rinsed three times with PBS, overlaid with biotinylated secondary Abs (1:200) (Vector Laboratories, Burlingame, CA), and incubated another 10 min, followed by three additional rinses with PBS. At this point, slides to be evaluated qualitatively were treated with peroxidase-labeled avidin, rinsed three times, then overlaid with substrate chromogen (3-amino 9-ethyl carbazole) and incubated for 15 min at 37°C to allow for color development. Mayer's hematoxylin was used as a counter stain. Those slides used for quantitative analysis were treated with fluorochrome-labeled avidin for 20 min, rinsed three times, counterstained, and mounted. Image analysis was then used to localize and quantify any bound fluorochrome.

Assay for cell-associated catecholamines

Adherent, monolayer Mϕs (approximately 8×10^{10} cells) were scraped in ice-cold sterile, serum-free RPMI 1640 and collected into a single pellet by centrifugation at 1000 × *g* for 10 min. Cell pellets were homogenized in 1 ml of 0.4 N perchloric acid ($HClO_4$) containing EDTA and sodium metabisulfite (30), centrifuged at 25,000 × *g* and supernatants were collected. Catecholamines in the cell extracts were measured by HPLC with electrochemical detection using a Waters plasma catecholamine analysis system and procedure (Waters Chromatography Division, Millipore Corp., Bedford, MA). Catecholamines were first adsorbed onto aluminum oxide at pH 8.7. After washing with water, the catecholamines were eluted with a dilute solution of acetic acid and EDTA. The resulting concentrated sample was injected onto a reverse phase C18 column and the eluant monitored by an electrochemical detector for the presence of the individual catecholamines.

Experimental protocol

The experiments were designed to establish whether or not the Mϕ-associated norepinephrine activation of α2- and β-adrenergic receptors regulates LPS-induced gene expression of TNF mRNA and production of the biologically active protein. Mϕs in monolayer were incubated in the presence or absence of adrenergic drugs plus single (100 ng/ml) or graded concentrations of LPS (*Escherichia coli* 0111:B4, Sigma). After 4 h incubation, or at specific time points for kinetic studies, cell-free supernatants were collected and stored at −20°C until bioassayed, whereas the cells were prepared for immunolocalization of TNF. In subsequent studies, treated cultures were terminated at 3 h for TNF mRNA analysis. In later experiments, Mϕ monolayers were stimulated with LPS (1 μg/ml) and terminated 30 min later for catecholamine content determination or washed extensively and allowed to remain in culture for an additional 12 h. After 12 h incubation, adrenergic sensitivity was determined.

Statistics

Complete concentration-effect relationships for LPS and isoproterenol were determined to establish changes in the TNF response. The percentage of response obtained at various LPS or isoproterenol concentrations was obtained by using nonlinear least squares regression to fit a curve. All data are expressed as mean values ± SE. Statistical significance was evaluated with a paired Student's *t*-test.

Results

Concentration-dependent effect of isoproterenol on Mϕ-derived TNF production after LPS stimulation

The production of TNF by LPS-stimulated peritoneal Mϕ was found to be concentration dependent between 10 pg and 100 ng. The EC_{50} for TNF production from Mϕ treated with LPS alone was 0.88 ± 0.24 ng/ml (data not shown). The β-adrenergic agonist, isoproterenol (10 μM), had an inhibitory effect on LPS-induced TNF production, as demonstrated by an eightfold parallel shift to the right

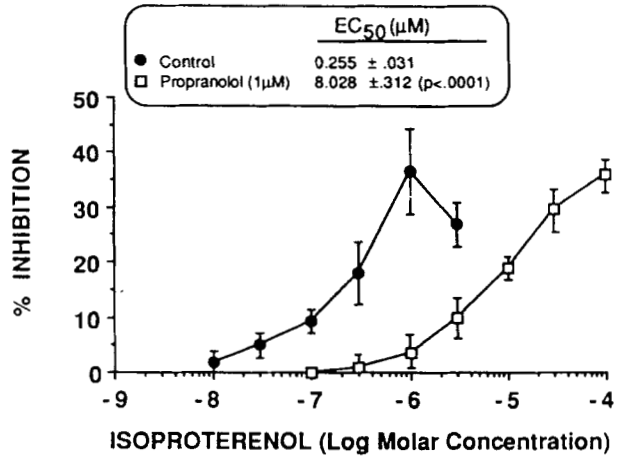

FIGURE 1. Inhibition of the release of TNF by isoproterenol during LPS stimulation of Mϕ and the antagonism of propranolol on the isoproterenol response. Inhibition of TNF released (310 U) from Mϕ in the presence of LPS alone. Mϕs were stimulated with LPS (30 ng/ml) alone and with various concentrations of isoproterenol and propranolol. Supernatants were assayed for TNF 4 h later. Each point represents five to eight determinations ± SE.

in the LPS concentration-effect curve to an EC_{50} of 7.03 ± 2.1 ng/ml ($p < 0.05$). Isoproterenol also inhibited LPS-stimulated TNF release in a concentration-dependent manner (Fig. 1). The EC_{50} for inhibition by isoproterenol of LPS-stimulated TNF release from Mϕ was 0.255 ± 0.031 μM. When the β-adrenergic antagonist propranolol (1 μM) was added along with isoproterenol there was a 31-fold shift to the right in the isoproterenol concentration-effect curve with regard to LPS-stimulated TNF release from Mϕ (Fig. 1). This was indicated by a significant increase in the EC_{50} for isoproterenol (8.028 ± 0.312 μM, $p < 0.0001$). The inhibition of TNF production by β-adrenergic receptor stimulation was demonstrable at the level of RNA expression, as shown in Figure 2. The simultaneous administration of isoproterenol with LPS led to a concentration-dependent reduction in TNF mRNA accumulation compared with LPS alone (Fig. 2). Furthermore, the inhibition of TNF mRNA accumulation by isoproterenol was competitively blocked by the simultaneous addition of propranolol.

Effect of adrenergic antagonists on Mϕ-derived TNF production after LPS stimulation

In contrast to the effect observed with the β-adrenergic agonist, the β-adrenergic antagonist propranolol augmented TNF production. In addition, the α-adrenergic antagonists, yohimbine and idazoxan, inhibit TNF production. The production of TNF by LPS-stimulated Mϕ was found to be dose dependent between 1 pg/ml and 100 ng/ml (Fig. 3). Adrenergic antagonists were added simultaneously to Mϕ with LPS to evaluate the autoregulatory

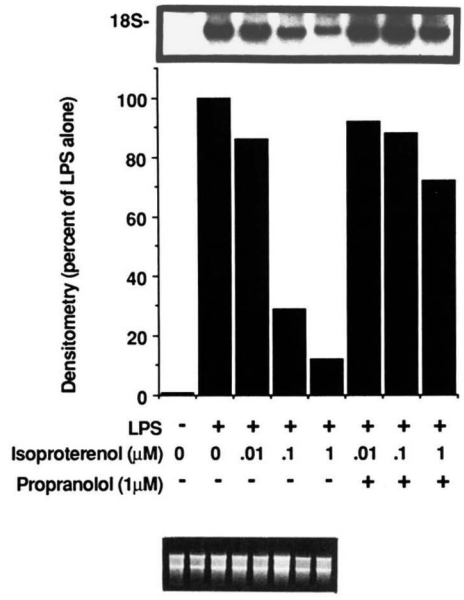

FIGURE 2. Inhibition by isoproterenol of LPS-induced TNF mRNA accumulation and the effect of propranolol on the isoproterenol response. Mϕs were treated with LPS (30 ng/ml) alone or in the presence of isoproterenol and propranolol for 3 h, and Northern analysis was performed on RNA extracts taken 3 h after LPS addition. The upper blot was probed for TNF-α and the lower blot represents 28s and 18s rRNA.

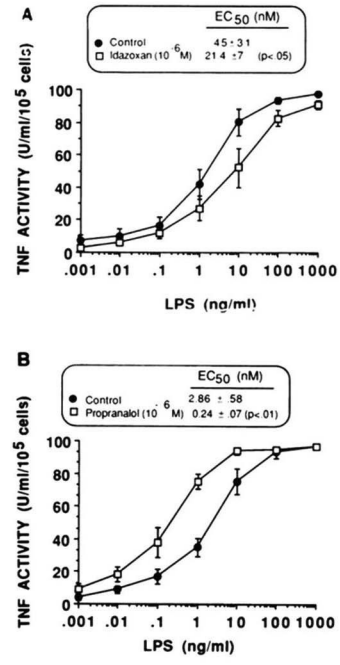

FIGURE 3. Effects of the α2-adrenergic antagonist idazoxan and the β-adrenergic antagonist propranolol on the concentration-effect curve of LPS. Mϕs were stimulated with LPS (1 pg/ml to 1 μg/ml) alone or in the presence of the adrenergic antagonists. Supernatants were assayed for TNF 4 h later. Each point represents the mean of four to seven determinations ± SE.

role of the adrenergic receptors on Mϕ. Shifts in the LPS concentration-effect curves were observed when antagonists were added to LPS stimulated Mϕ. The α2-adrenergic antagonist, idazoxan, inhibited LPS-induced TNF production (Fig. 3*A*). The LPS concentration-effect curve was significantly shifted to the right from an EC_{50} of 4.5 ± 3.1 ng/ml for LPS alone to 21.4 ± 7 ng/ml ($p < 0.05$) for LPS in the presence of idazoxan (10^{-6} M). In contrast, although the EC_{50} for LPS-induced TNF release was 2.86 ± 0.58 ng/ml, the addition of the β-adrenergic antagonist, propranolol, produced a parallel shift to the left (0.24 ± 0.07 ng/ml, $p < 0.01$; Fig. 3*B*).

Adrenergic antagonists were also found to alter LPS-stimulated TNF release in a concentration-dependent manner (Fig. 4). Propranolol increased TNF release and the α2-adrenergic antagonist, yohimbine, inhibited TNF release. The simultaneous administration of propranolol with LPS increased TNF mRNA accumulation, and the simultaneous administration of yohimbine with LPS decreased (Fig. 5) TNF mRNA accumulation, as assessed by Northern blot analysis. These results were at concentrations similar to those observed for regulating the release of bioactive TNF.

Catecholamine content of Mϕ populations before and after LPS stimulation

In our studies, Mϕs were demonstrated to have stored catecholamines and, on LPS stimulation, those stored levels were significantly decreased. Control Mϕs were found to possess 624 ± 120 pg/10^8 cell of norepinephrine, 192 ± 38 pg/10^8 cell of epinephrine, and 284 ± 58 pg/10^8 cell of dopamine. LPS (1 μg/ml, 30 min) stimulation decreased the norepinephrine content (385 ± 60 pg/10^8 cell, $p < 0.05$, $n = 6$) and the epinephrine content (95 ± 30 pg/10^8 cell, $p < 0.05$, $n = 6$). LPS challenge of Mϕ did not change the content of dopamine (312 ± 74 pg/10^8 cell, NS, $n = 6$).

Effect of UK-14304 and yohimbine on LPS-stimulated accumulation and release of TNF and relationship to norepinephrine depletion

The effect of α2-adrenergic receptor stimulation on TNF production was investigated during the first 30 min after LPS exposure (100 ng/ml). Bioassay and immunohistochemical analysis were used to assess TNF production. Figure 6 demonstrates the immunohistochemical detection of cell-associated TNF. As shown in Figure 6*A*, Mϕ contained TNF, as detected by immunohistochemical stain, 30 min after LPS stimulation. Although the α2-adrenergic agonist had no effect on cell-associated TNF expression (Fig. 6*C*), the TNF expression decreased in the presence of the α2-adrenergic receptor antagonist, yohimbine (Fig. 6*B*). This effect was prevented when the specific agonist was added with yohimbine (Fig. 6*D*).

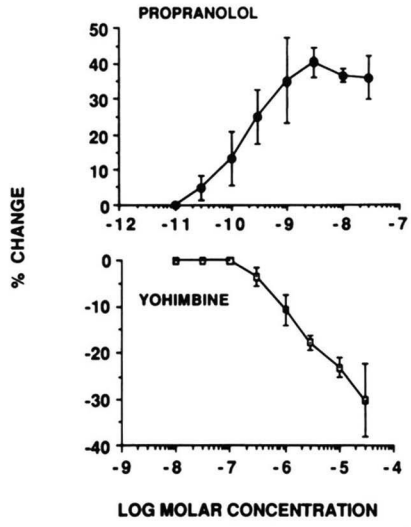

FIGURE 4. Concentration-dependent effects of the α2-adrenergic antagonist yohimbine and the β-adrenergic antagonist propranolol on LPS-induced TNF production. Mϕs were stimulated with LPS (30 ng/ml) alone or in the presence of the adrenergic antagonists. TNF levels were assessed 4 h after the addition of the above compounds. Each point represents the mean of six determinations ± SE.

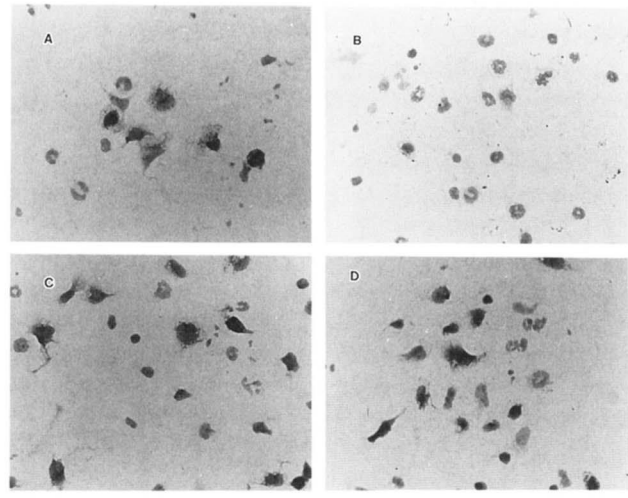

FIGURE 6. Immunohistochemical localization of TNF in cultured Mϕ and the effect of α2-adrenergic drugs on TNF content. Mϕ monolayers were stimulated with LPS (100 ng/ml) and stained for TNF 30 min later using specific polyclonal Abs. *A*: LPS control. *B*: LPS plus yohimbine (1 μM). *C*: LPS plus UK-14304 (100 nM). *D*: LPS plus UK-14304 and yohimbine. Magnification ×150.

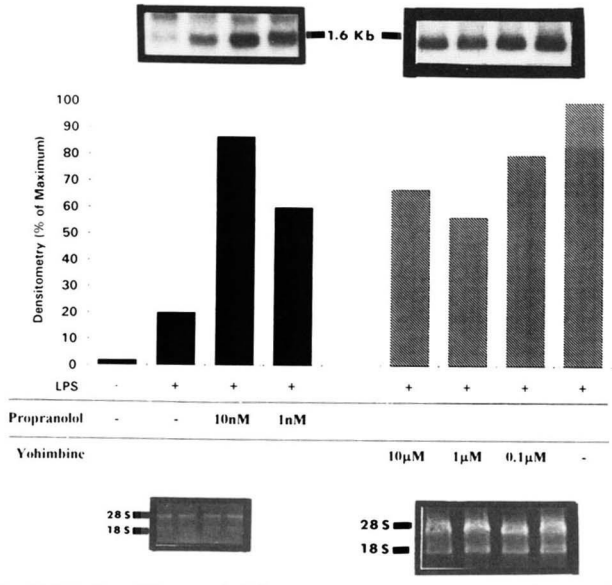

FIGURE 5. Effects of different concentrations of adrenergic antagonists on LPS-induced TNF mRNA accumulation. Mϕs were treated with LPS (10 ng/ml for propranolol or 100 ng/ml for yohimbine effect) alone or in the presence of different concentrations of propranolol or yohimbine. Northern analysis was performed on RNA extracts taken 3 h after LPS addition. The upper blot was probed for TNF-α and the lower blot represents 28s and 18s rRNA.

Expression of LPS-induced TNF by cultured, naive peritoneal Mϕ occurred within 10 min and continued through 30 min (Fig. 7). The effect of α2-adrenergic receptor stimulation on the LPS response was not observed at these early time points, but blockade of α2-adrenergic receptors by the appropriate antagonist prevented TNF production. The agonist (UK-14304) could prevent the inhibition by the antagonist of cell-associated TNF but did not reverse the effect of the antagonist on the release of TNF. This may be due to different levels of regulation of TNF production between cell-associated TNF and the release of biologically active TNF, and therefore different potencies of the adrenergic compounds. Interestingly, the adrenergic responses were reversed in cells that were pretreated with LPS (Fig. 8). Expression of TNF at early time points after LPS stimulation were also studied in Mϕ that were previously (12 h) stimulated with LPS (1 μg/ml, 30 min) and then washed. The effect of α2-adrenergic receptor stimulation on the LPS response was also determined in Mϕ that had been previously exposed to LPS (Fig. 8). In these studies, LPS-pretreated Mϕs that were subsequently rechallenged with LPS demonstrated a decreased response. This was demonstrated by a delay in responsiveness and a decrease in the efficacy of LPS. In contrast to naive cells, UK-14304 increased the LPS-induced TNF response, an effect that was reversed by the antagonist, whereas the antagonist had no effect when added alone with LPS.

Discussion

Neuroendocrine hormones are among the endogenous mediators that may potentially influence Mϕ responsiveness. They originate from the nervous system and are integrated into numerous biologic responses including those of immune cells. Therefore, much speculation exists that autonomic endocrine hormones participate in neuroimmune communication. Many studies have demonstrated that the

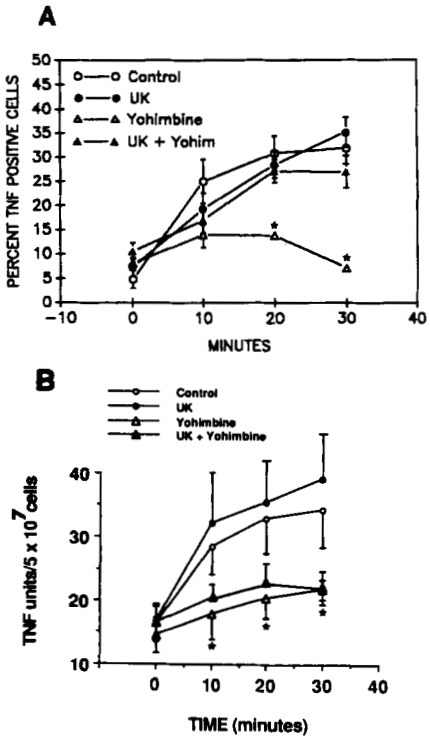

FIGURE 7. Expression of TNF by cultured naive peritoneal Mϕ at early time points after LPS stimulation, and the effect of α2-adrenergic receptor stimulation on the LPS response. *A*: Percentage of TNF-positive cells obtained by quantitating positive immunohistochemical staining patterns in cultured Mϕ. Mϕs were stained for TNF-α at time 0, 10, 20, and 30 min after LPS (100 ng/ml) stimulation. *B*: Kinetics of TNF-α production (WEHI assay) by cultured Mϕ during LPS stimulation (100 ng/ml). All points are the mean ± SE for three to six determinations.

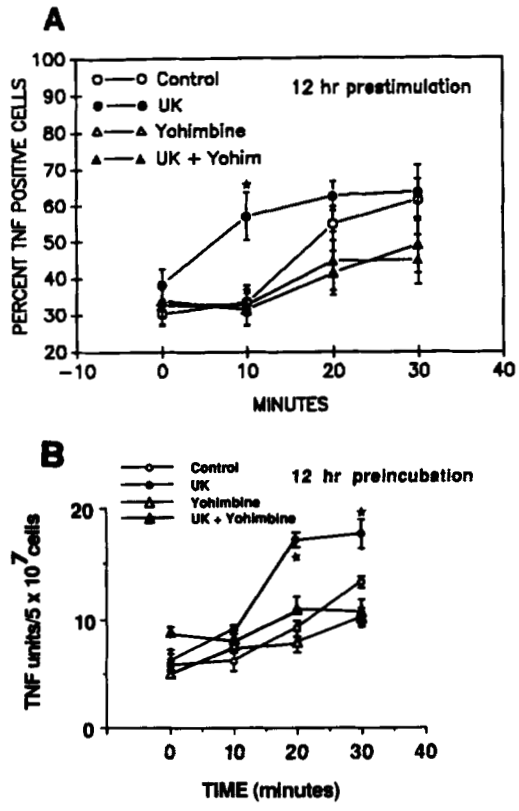

FIGURE 8. Expression at early time points after LPS stimulation of TNF by cultured peritoneal Mϕs that were previously (12 h) stimulated with LPS (1 μg/ml, 30 min) and washed extensively. As in Figure 7, the effect of α2-adrenergic receptor stimulation on the LPS response was determined. *A*: Percentage of TNF-positive cells obtained by quantitating positive immunohistochemical staining patterns in cultured Mϕ. Mϕs were stained for TNF-α at time 0, 10, 20, and 30 min after LPS (100 ng/ml) stimulation. *B*: Kinetics of TNF-α production (WEHI assay) by cultured Mϕ during LPS stimulation (100 ng/ml). All points are the mean ± SE for three to six determinations.

neuroimmune communication network is actively engaged during inflammatory responses and stress (1–5). In particular, adrenergic compounds have been shown to regulate Mϕ responsiveness (6–10, 16–20). Compounds that act via β-adrenergic receptors reduce the activity of immunocompetent cells. In contrast, stimulation of cells via α-adrenergic receptors results in an augmentation of responsiveness of immunocompetent cells. The catecholamine norepinephrine augments Mϕ phagocytosis and tumoricidal activity at concentrations that are similar to those previously reported for stimulating the α-adrenergic receptor (29).

We have previously demonstrated that Mϕs possess a functional α2-adrenergic receptor that binds the α2 antagonist yohimbine in a rapid, saturable, and reversible manner (17). In addition, stimulation of the Mϕ α2-adrenergic receptor with either norepinephrine or UK-14304 significantly increases LPS-induced Mϕ TNF production, with an apparent affinity similar to that obtained in binding. It has also been demonstrated that stimulation of β-adrenergic receptors on Mϕ causes an inhibition of LPS-stimulated TNF production (16). This previous study illustrates that stimulation of β-adrenergic receptors on human blood monocytes and THP-1 cells results in the inhibition of LPS-stimulated TNF production. These previous investigations not only demonstrate an inhibition of TNF production but also show an increase in cAMP production by β-adrenergic receptor stimulation of THP-1 cells. In addition, they show that β-adrenergic receptor stimulation does not result in changes in TNF mRNA accumulation.

This study demonstrates that the β-adrenergic agonist isoproterenol, in a concentration-dependent manner, inhibits its Mϕ-derived TNF mRNA accumulation and the release of bioactive TNF. Because it has been documented that the mechanism of suppressive effects of mediators that inhibit LPS-stimulated TNF production can occur via the generation of intracellular cAMP (30, 31), the apparent discrepancy between this study and the previous investigation may be due to a characteristic unique to the THP-1 cell line. It has now become apparent that the magnitude of the

effects of adrenergic agonists on Mϕ-derived, LPS-stimulated TNF production is more than modest. In addition, because neuroendocrine hormones play a role in numerous biologic responses a better understanding of adrenergic regulation is worthy of consideration.

This study reveals an interaction between a neurotransmitter and the immune system in regulating the expression of Mϕ-derived TNF production. Norepinephrine can act through two different opposing receptors on CFA-elicited Mϕ, presumably depending on the concentration of the neuroendocrine hormone. This study was designed specifically to examine activated Ia^+ Mϕ as might be found in vivo in a disease setting. Because it has already been demonstrated that CFA-elicited Mϕs are responsive to α- and β-adrenergic drugs, this population was chosen to investigate the role of norepinephrine as an autocrine mediator. Whether other Mϕ populations use a pool of endogenous norepinephrine to regulate cytokine production is included in future studies. These investigations could help explain how a lesion is maintained when levels of other mediators, such as PGE_2, are increased at inflammatory foci (32, 33). Because this study demonstrates that the modulation of Mϕ-derived TNF production by norepinephrine is an extremely dynamic process, it could further explain the neuroimmune modulation that may induce altered physiology in populations of stressed individuals (34).

This study (Figs. 7 and 8) demonstrates an increase in TNF-α protein that occurs within the first 30 min after LPS stimulation of Mϕ. This amount of TNF-α production is small compared with the amount observed at the 4- to 6-h peak production (many more cells are needed to observe the response). Because the early release of TNF is a very small amount that appears to plateau by 30 min, we believe this is an example of preexisting mRNA, which is independent of that which occurs at 4 to 6 h. Although this early release of TNF produces a very small quantity of TNF compared with the total amount the Mϕ is able to produce, the regulation of its production by adrenergic receptors leads us to believe it represents an important pool. Furthermore, not only do adrenergic receptors regulate the production of this early pool of TNF, but they also regulate that which is produced at the 4- to 6-h time point (Figs. 1 to 5). In addition, if the total amount of norepinephrine in the Mϕ was released on activation and fully diluted in the supernatant, it would reach low, nanomolar concentrations.

Low nanomolar concentrations of norepinephrine are around the EC_{100} for the norepinephrine concentration-effect curve for the α-adrenergic regulation of LPS-stimulated TNF release (17). Not only can a low nanomolar concentration of norepinephrine achieve some responses at the β-adrenergic receptor but in addition, as we have demonstrated in this study, the catecholamine epinephrine is also released and has a higher affinity at the β-adrenergic receptor. Furthermore, low nanomolar concentrations are reached only after full dilution in the supernatant, what is achieved transiently at local environments can only be greater. Because all the data presented demonstrates adrenergic receptor activation on LPS stimulation, we believe appropriate concentrations of the catecholmines are reached to stimulate both receptor populations.

This paper presents data to demonstrate for the first time that Mϕ contain norepinephrine and use it during a physiologic response. The fact that specific adrenergic antagonists when added alone to LPS-stimulated Mϕ produce a response that is opposite to that generated by the addition of the appropriate agonist demonstrates the role of the endogenous agonist. Furthermore, the shift by the antagonists are significant with a 4.8-fold shift by the α-adrenergic antagonist (when an EC_{30} concentration was used; Fig. 4) and a 11.9-fold shift by the β-adrenergic antagonist. In addition, Figure 4 demonstrates that both antagonists are functioning in a concentration-dependent manner and at concentrations that are consistent with an adrenergic response for these antagonists. Finally, Figures 6 to 8 demonstrate that after depletion of the endogenous pool of catecholamines (significantly depleted after LPS stimulation, as demonstrated by HPLC in the text), the response to agonists and antagonists is altered, an effect that may help explain LPS tolerance. These changes in adrenergic responsiveness can best be explained only by the depletion of an endogenous pool of catecholamines.

Because it has been established that the Mϕ is a site for extraneuronal uptake of catecholamines (23, 35), it was our interest to determine whether there is a physiologic role for this accumulation. To the best of our knowledge, it has never been demonstrated that Mϕs have and use catecholamines in an autoregulatory fashion. Furthermore, it has never been demonstrated that the pool of catecholamines, at least in part, influences the responsiveness of the Mϕ to LPS. This study further addresses the physiologic effect of α2-adrenergic and β-adrenergic receptors on Mϕ. Complex mechanisms involving these receptor populations would support the concept that communication circuits between Mϕs and other physiologic signals are active processes. The ability to regulate the production of TNF due to stimulation of Mϕ by LPS is not only dependent on Mϕ receptor populations, but also on its own stores of endogenous autoregulatory mediators. Not only do Mϕs have a pool of endogenous norepinephrine, but when stimulated by LPS, they are able to release this pool and potentially autoregulate TNF production.

Because it has been reported that Mϕ have a low affinity norepinephrine uptake system (23, 35) it would follow that not only does it take considerable time to accumulate catecholamines, but once released they could permanently alter the Mϕ responsiveness. Indeed, this study demonstrates that once this pool is depleted after LPS stimulation, the responsiveness of the adrenergic receptors is changed. Although this may not by itself explain Mϕ tolerance to LPS, it may play an important role. Although it appears that adrenergic receptors alone do not dictate Mϕ

tolerance to LPS, future studies may further define the role of these receptors. Our data may provide better insight into other ways to control TNF production in the coordination of homeostasis, stress, and disease. Furthermore, we believe this is the first demonstration of the utilization of norepinephrine by cells outside the nervous system. Although previous studies have demonstrated that Mϕ can accumulate extraneuronal norepinephrine (23, 35), this is the first report identifying a regulatory role for this pool of neurotransmitter.

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