STUDYING MODULATION OF TUMOR NECROSIS FACTOR-α AND TRANSFORMING GROWTH FACTOR-β BY NOREPINEPRHINE IN MURINE ALVEOLAR MACROPHAGES, MURINE SPLENOCYTES AND TRANSFORMED CELL POPULATIONS

by

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B.Sc. Honours, Acadia University, 1996

Thesis
submitted in partial fulfillment of the requirements for
the degree of Master of Science (Biology)

Acadia University
Fall Convocation 1998

ACKNOWLEDGMENTS

Thank-you Dr. Hilda Taylor and Dr. Glenys Gibson for serving on my advisory committee, for your valuable insight, for your encouragement and also for your guidance. A very special thank you to Dr. Colin Bell for assuming the role of supervisor so late into my Masters, and for the guidance and support he offered throughout the final stages of this project.

Special thank-yous to Wanda Langley, Jeanette Simpson and Dr. Tom Herman for their time and personal guidance. To Hélène d'Entremont - thank you for listening, being my friend, and always finding a rainbow even at the end of the most horrible storms. To Carol and Jim Legge, a special thank you for the awesome times away from the lab, and for providing me with a home away from home. You both have shown me that when I get knocked down, I can and do get back up again. Also, a very special thank-you to Canon Hergett for providing me with guidance throughout this experience, but even more importantly, thank your for your treasured friendship.

Thank you to NSERC and Acadia Graduate Studies for the monetary support of my research and my salary, and to Archie McNichol for the generous donation of UK14304. Much appreciation is also given to Dr. Daryl Grund and again to Dr. Colin Bell for the use of their laboratory, equipment and supplies.

Finally, thank you Mom and Dad for all your love and support through this tremendous learning experience, paying for all those long-distance phone calls, for your encouragement, and most of all -- for always believing in me, even when I didn't.

"Why are there so many songs about rainbows and what's on the other side?
Rainbows are visions, but only illusions, and rainbows have nothing to hide.
So we've been told and some choose to believe it - I know they're wrong, wait and see.
Someday we'll find it, the rainbow connection, the lovers, the dreamers and me."
-Kermit the Frog

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BAL	bronchoalveolar lavage
CD	cluster of differentiation
FBS	fetal bovine serum
IL-6	interleukin-6
IPF	idiopathic pulmonary fibrosis
kDa	kilodalton
LPS	lipopolysaccharide
mAb	monoclonal antibody
ME	methoxamine
MHC	major histocompatibility complex
MP	mononuclear-phagocytic
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
NE	norepinephrine
NPH	norepinephrine and phentolamine (1:1 ratio)
NPR	norepinephrine and propranolol (1:1 ratio)
OD	optical density
PH	phentolamine
PMA	phorbol myristate acetate
PR	propranolol
rTGF-β	recombinant transforming growth factor-β
rTNF-α	recombinant tumor necrosis factor-α
TCR	T cell receptor (for antigen)

TE	terbutaline
TGF-β	transforming growth factor-β
Th1	T helper lymphocyte, type I
Th2	T helper lymphocyte, type II
TNF-α	tumor necrosis factor-α
UK	UK 14304

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ABSTRACT

The involvement of the nervous system in modulating immune function comprises the exciting field of neuroimmunology. Not only has it been proven that the nervous system can participate in controlling immune function in a variety of illnesses and diseases, but scientists have also demonstrated that the immune system is able to regulate the nervous system. Extensive research has identified at least one catecholamine, norepinephrine, which is able to participate in the cross-talk between these two systems. Norepinephrine, a normal constituent of the mammalian brain, is able to interact with postsynaptic receptors to mediate a diverse set of biological responses when it is released from nerve endings. These receptors, as well as being located on nervous system tissues, have also recently been found on cells of the immune system, including macrophages and lymphocytes. Most research to date has focused on the effects of norepinephrine on splenic macrophage function and these studies have noted that norepinephrine is able to modulate a diverse set of immune activities, depending upon the cellular targets and the concentration of this catecholamine. Although researchers have illustrated that norepinephrine has an effect on the modulation of tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) in splenic macrophage populations, research has yet to determine the effect of norepinephrine on the production of these cytokines by lymphocytes or by LPS stimulated and non-stimulated murine alveolar macrophages. Utilizing four lymphocyte cell systems, two transformed cell lines and both non-stimulated as well as a CD3 + PMA stimulated murine splenocytes, it was concluded that norepinephrine was able to significantly increase production of both cytokines by the first three cell systems, but the modulatory effect of norepinephrine on the CD3 + PMA stimulated splenocyte sample was significantly inhibitory. These effects of norepinephrine were noted to reflect both the concentration and adrenoceptor target of the adrenergic drug. The murine alveolar macrophage populations displayed similar results, with norepinephrine again being able to modulate adrenoceptor-specific increases and decreases in TNF-α and TGF-β production. The cytokines, both TNF-α and TGF-β, were quantified throughout this research project using a non-radioactve MTT bioassay method specifically designed in this Masters Project. The results of this experimental research project are meaningful in elucidating the role of the nervous system in immune function, but also relate more importantly to the involvement of norepinephrine in cytokine modulation in diseases, including idiopathic pulmonary fibrosis, chronic lung disease and many forms of cancer.

CHAPTER I:

AN OVERVIEW

INTRODUCTION

Traditionally, the immune system has been regarded as an autonomous, self-regulating collection of specialized cells and organs which have the ability to identify and clear elements and microorganisms foreign to the host (1, 2, 3). In the early 1930s, the first hint of a connection between the immune system and nervous system was established when Hans Selye demonstrated that stress could potentiate both hypertrophy of the adrenal glands and atrophy of the thymus (3). Moreover, Selye also illustrated that adrenalectomy, or hypophysectomy, substantially reduced these stress induced physiological changes (3). Selye's experimental evidence suggested that the thymus is sensitive to stress, and that a link between the immune system and stress may be potentially mediated through the pituitary-adrenal endocrine axis (3).

More recently, in the early 1980s, evidence was published illustrating that complex interactions occurred regularly between the brain and the immune system (2, 4, 5, 6, 7, 8). Rinner examined the myriad of factors originating in both the central nervous system and the endocrine system and found that the immune system, indeed has the potential to respond to these changes (2, 3, 6, 7, 8). With this new evidence, and that previously of Selye, the field of psychoimmunology became recognized, and today has grown to become a rapidly expanding field in modern biology.

THE IMMUNE SYSTEM AND NERVOUS SYSTEM -- A RELATIONSHIP?

Interactions between the immune system and psychological states have been recognized as both intricate and intriguing (9). Researchers have shown through various psychosocial, biological and neurobiological studies that multiple channels of communication exist between the central nervous system and the immune system (2, 6, 9, 10, 11, 12, 13). In fact, the cross-talk involving both systems has been largely established through numerous experiments examining both the physical and chemical connections between these two systems (2, 6, 9, 10, 12, 13, 14, 15, 16). It has been demonstrated that both these systems use similar ligands and receptors to establish a physiological intra- and interstem communication circuitry (2, 6, 13, 16, 17). This circuitry plays a relevant and important role in maintaining homeostasis within the body (2, 6, 9, 10, 13, 15, 16, 18).

More evidence, supported by the observations that both primary and secondary lymphoid organs and tissues are richly innervated by noradrenergic sympathetic fibers, suggested the possibility of further physical and chemical links between these two systems (6, 10, 11, 13, 17, 18, 19). Nerve terminals are concentrated, and in direct contact with lymphocytes in zones of lymphoid tissues rich in T cells -- including the thymus, and in the bone marrow -- where B cells develop (7, 10, 13, 18, 19, 20). The anatomical compartmentalization of noradrenergic innervation in lymphoid organs suggests the existence of sympathetic neural interactions, more specifically through the neurotransmitter norepinephrine (NE) (7, 10, 13, 18, 19). Indeed, evidence for such interactions came from work by Bishopric and Cohen (19) who discovered the expression of high affinity β-adrenoceptors on T and B lymphocytes as well as on macrophages (10, 13, 15, 19). The presence of α-adrenoceptors on human lymphocytes has been comprehensively documented by ligand binding studies completed by many scientists including, Titinchi and Clark (4, 10, 13). These adrenoceptors, like their counterparts found on other tissues innervated by the sympathetic nervous system (SNS), are regulated

by the nervous system, with their receptors being downregulated in the presence of agonists, and upregulated in the presence of β-blockers (10, 13, 15).

It has been found that *in vitro*, adrenergic agonists can modulate all aspects of an immune response, altering such functions as cytokine production, lymphocyte proliferation and antibody secretion (10, 11, 13, 14, 16, 20, 21). *In vivo*, it has been observed that chemical sympathectomy suppresses cell-mediated responses and may in fact enhance antibody responses (10, 11, 13, 15, 20). Likewise, noradrenergic innervation of the spleen and lymph nodes is diminished progressively during chronic stress, a time when cell-mediated immune function is also suppressed (10, 13, 20, 21). In animal models of autoimmune disease, sympathetic innervation is reduced prior to the onset of disease symptoms, and chemical sympathectomy can exacerbate disease severity (10, 13, 20, 21).

A basis for the neural modulation of immunity is suggested by this experimental evidence, and presents a potential role for the nervous system in the development of the immune system (6, 10, 12, 13, 22). These findings illustrate the importance of the sympathetic nervous system in modulating immune function under both normal and disease states (4, 6, 10, 12, 13, 14, 20, 23).

THE IMMUNE CELL POPULATION

All the cells that participate in an immune response originate from a population of cells known as stem cells (24, 25, 26). Stem cells are able to reproduce and give rise to special cell populations like neutrophils, macrophages and lymphocytes (26). Lymphocytes are the key cells of the immune system since they recognize and respond to foreign antigens while macrophages are valuable both as phagocytic cells and for their role in antigen presentation (25, 26).

The mononuclear-phagocytic (MP) system consists of a single population of cells called macrophages (25, 26). Macrophages of the MP system are located in many locations including the lungs, liver, spleen, lymph nodes, and blood (25, 26). They have a

variety of functions in these locations, some of which are phagocytosis, macrophage activation, the generation of reactive nitrogen metabolites, and they serve as important secretory cells (25, 26). Almost 100 types of proteins are secreted by macrophage cells including enzymes, leukotrienes and cytokines (26). Of the cytokines, five are prominent in the regulation of immune responses -- these include interleukin-1, interleukin-12, interleukin-6, tumor necrosis factor-α and transforming growth factor-β (25, 27).

Lymphocytes are extraordinarily complex cells, found in tissues and fluids throughout the body, including the blood and such organs as the spleen, thymus and lymph nodes (4, 26). The immune response primarily takes place in these areas where lymphocytes are focused, therefore the blood and lymphoid organs prove important in establishing an environment for efficient interactions between antigen, lymphocytes, and antigen presenting cells -- B cells and macrophages (25, 26). Lymphocytes are characterized by surface receptors, surface proteins, their response to mitogens, and developmental origin (4, 26, 28). Those cells that develop in the thymus are known as T cells, whereas B cells mature in the bone marrow (25, 26). While B cells can recognize antigen in body tissues and fluids, T cells can recognize only selected fragments of antigen (25, 26, 28). There are two major divisions of T cells based on antigen presentation and recognition (226, 29). T lymphocytes that develop into cytotoxic T cells recognize endogenous antigen presented with a major histocompatibility complex (MHC) class I molecule (26, 29). Exogenous antigen presented bound to a major histocompatibility complex (MHC) molecule of the class II type is recognized by the second type of T cells -- helper T cells (26, 29). The other characteristic defining these two groups of T cells is the presence or absence of the accessory molecules, CD4 and CD8. T cells with CD8, but lacking CD4, bind only to antigen presented on the surface of virus-infected or other altered cells -- recognizing the MHC class I molecule, while T cell populations with CD4, but lacking CD8, will bind only to the MHC class II molecule on antigen presenting cells (26, 29). These two molecules, CD4 and CD8, therefore form receptor complexes which

bind to conserved structures on the MHC molecules and thus have major roles in regulating T-cell response (26, 29).

The T helper (Th) lymphocyte group can further be subdivided into T helper 1 and T helper 2 subclasses based on cell function and patterns of cytokine production (29, 30, 31, 32). T Helper 1 (Th1) cells secrete predominantly interferon-γ (IFN-γ) and IL-2, whereas T helper 2 (Th2) cells secrete mainly IL-4, IL-5 and IL-13 (29, 31, 32, 33, 34, 35). Th1 cells are able to induce cellular mediated inflammation and tissue injury whereas Th2 cells are prominent in the pathogenesis of allergic diseases (28, 29, 31, 32). The cytokines which are present during the initiation of a T cell response also play a role in determining the development of a particular subset of T helper lymphocytes (27, 28, 29, 32). Th2 cells develop when naive T cells are stimulated in the presence of IL-4 (29, 32). Conversely, IL-12 is a critical factor driving the development of Th1 cells (28, 29, 32).

CYTOKINES

In recent years, the understanding of the immune system has expanded dramatically with increased knowledge of the individual components of the system and how these components interact to produce local and systemic inflammatory responses (2, 22, 36, 37). The coordination of the different cell types involved in inflammatory responses occurs through various pathways, including humoral mediators -- cytokines (2, 22, 36). These cytokines, although relatively specific in their targets and actions, frequently have overlapping functions (2). Recent evidence has suggested that some cytokines are able to activate elements of the hypothalamic-pituitary-adrenal system and thus are directly able to elicit immune responses, including fever (2, 38).

As a result of appropriate stimulation, cells of the immune system are able to secrete a tremendous number and wide variation of proteins that mediate signaling between cells (22, 26, 39). Interleukins are cytokines that either enhance interactions between leukocytes and other lymphocytes, or serve as cell growth stimulants (26). An

example of a cytokine is interleukin-1, secreted mainly by macrophages in response to a wide variety of stimuli, including phagocytosis or direct contact with T lymphocytes (26, 39, 40). Interleukin-6 is secreted mainly by T cells and fibroblasts and has a variety of major targets including B cells (26, 39, 40). IL-6 is able to promote IL-2 production and also is capable of stimulating the differentiation of T cells (26). Tumor necrosis factors are produced both by macrophages and T cells, and they have the ability to kill tumor cells (26). Finally, transforming growth factor-β is produced by macrophages, T cells and B cells and is involved in a variety of cellular processes including, proliferation, differentiation, and immunoglobulin production (26, 36, 41, 42, 43, 44).

Cytokines not only play a major role in local tissue response to microbial invasion, but they are also significant in the systemic effects of many diseases (26, 36, 45, 46). Fever is one of the best examples of a neuroimmune response to disease (47). Cytokines, such as IL-1, IL-6 and TNF-α, function as the main mediators of fever acting locally at the site of infection, and also circulating to the brain to modify body temperature and to control central nervous system response (2, 36, 37, 35, 47). Other diseases, including many cancerous tumors, are clinically recognized by abnormal levels of cytokines, and in some cancers, cytokines are known to increase metastasis rate and disease progression (46, 48, 49).

NOREPINEPHRINE -- THE NEUROTRANSMITTER

There are numerous substances that are either suspected or known neurotransmitters that can facilitate, inhibit or excite postsynaptic neurons (14, 50, 51). Norepinephrine belongs to this class of compounds, and is thus known as a catecholamine neurotransmitter (50, 51, 52). Catecholamines are a group of organic compounds that contain both an amine group and a catechol nucleus (50, 51, 52).

Shortly after norepinephrine was recognized as a neurotransmitter substance of adrenergic nerves in the peripheral nervous system, it was also classified a normal

constituent of the mammalian brain (23, 50, 51, 52). The relative distribution of norepinephrine is quite similar in most mammalian species, with the highest concentration being found in the hypothalamus and other areas of central sympathetic organization (50, 51, 52, 53, 54).

Norepinephrine, when released by nerve terminals of the sympathetic nervous system, is able to interact with postsynaptic receptors to mediate many diverse biological responses (4, 14, 50, 51). Once released, norepinephrine can stimulate either or both of the surface β-adrenergic or α-adrenergic receptors on effector cells (50, 51). The β-adrenoreceptor is coupled to the GTP-binding protein of the adenylate cyclase complex and thus, gives rise to increased intracellular cyclic AMP (cAMP) (20, 25, 38, 55, 56, 57). Phosphatidylinositol turnover and a rise in intracellular calcium levels is induced when α_1-adrenoceptors are stimulated, while α_2-adrenoceptors are linked to the G1 subunit of the adenylate cyclase complex (20, 25, 38, 56).

α-adrenoceptors have not as yet been identified on naive rodent lymphocytes, although they have been found to be expressed on naive human lymphocytes (20, 50, 58, 59, 60, 61). The expression of both α and β-adrenoceptors on activated rodent macrophages and human lymphocytes has been discussed in various research publications, however no studies on α-adrenoceptor density have been reported (20, 50, 55, 58, 60, 61). β-adrenoceptor density varies among species and their respective lymphocyte populations and has also been noted to fluctuate throughout cell development and differentiation (20, 58, 60, 61). β-adrenoceptors are present on thymocytes early in gestation and their intensity steadily increases with maturation; as yet, there remains no further research on this phenomenon (20, 20, 60, 61).

As mentioned, β-adrenoceptor density varies among lymphocyte populations and has been shown to fluctuate with activation by antigen (20, 50, 60, 61). For example, splenic B cells express twice the number of β-adrenoceptors expressed by T cells, although an explanation for this event has not yet surfaced (20, 50, 60, 61). In humans, T

helper cells have a low density of these receptors although the number of these receptors has been noted to increase with stimulation (20, 50, 60, 61). This difference in β-adrenoceptor concentration expressed by T and B lymphocytes is likely related either to the sparseness of innervation of B cell dependent follicles in the spleen, or may reflect heterogeneity within the T or B lymphocyte populations (11, 20, 50, 60, 61). Another interesting illustration regarding the concentration of β-adrenoreceptors reflects the subpopulations of T cells -- namely T helper 1 cells and T helper 2 cells (20, 50, 60). It has been found that resting Th1 and Th2 cells differentially express the β-adrenoceptor; β-adrenoceptors with high affinity have been detected on resting Th1 cells, but have not been found to be present on Th2 cells (20, 50, 60). In addition to this research by Sanders and Street, it has been noted that Th1 cells accumulate intercellular cAMP upon exposure to β-adrenergic agonists whereas Th2 cells do not (20, 50, 60). This discovery alone may provide a basis for the modulation of the Th cell subset by norepinephrine (20, 50, 60).

The adrenoceptor system involved in neuroimmunomodulation is a very complex system involving four primary adrenoceptor subtypes -- the α_1-, α_2-, β_1- and β_2-adrenoceptors. This subdivision of catecholamine receptors into alpha and beta receptors was initially proposed in order to distinguish the different physiological responses elicited by various organ systems after exposure to catecholamines (50, 60). Researchers studying adrenoceptor-induced modulation of immune function have since determined the adrenoceptor specific target of a series of adrenergic agonists and antagonists which ultimately are utilized to identify the adrenoceptor subtype responsible for the neuroimmunomodulation of a lymphocyte response. Currently, a variety of adrenergic agonists and antagonists are known for their adrenoceptor specificity. Norepinephrine is recognized as an α_1-, α_2-, β_1-, β_2-adrenergic agonist while terbutaline, although it has affinity for both β_1- and β_2-adrenoceptors on lymphocytes, has efficacy for primarily the β_2-adrenoceptor (50). To study the α-adrenoceptor effects of norepinephrine, two adrenergic agonists are known to have α-adrenoceptor specific affinity -- methoxamine

binds to the α_1-adrenergic adrenoceptor while UK14304 binds to the α_2-adrenergic adrenoceptor. Finally, adrenergic antagonists are used to block the various adrenoceptor specific effects in neuroimmune experimental studies -- propranolol serves as a β-adrenoceptor antagonist and phentolamine is recognized to block the α-adrenoceptor, thus blocking α-adrenoceptor modulation.

THE EFFECTS OF STRESS ON IMMUNE SYSTEM FUNCTION

Stress induced by environmental and psychosocial factors has been conclusively determined to suppress immune responses in humans (2, 7, 10, 23, 62, 63, 64, 65, 66). Studies have illustrated that cutting the splenic nerve significantly decreases splenic norepinephrine levels by 98-100% and essentially blocks the effects of stress (10, 23, 64, 66). From this research, not only is it apparent that norepinephrine levels are increased during stressful situations, but numerous studies have documented that these physical, chemical, or social stressors play an influential role in determining immunologic functions (10, 23, 64).

There are various research papers examining the changes in immune-inflammatory responses due to adrenergic receptor-activating substances like norepinephrine (10, 53, 54, 52, 64). Overall, these studies have documented both suppression and enhancement of immune responses as a direct result of selective stimulation of distinct adrenergic receptor populations (10, 53). These studies have also noted that norepinephrine has diverse activities, depending on the cellular target and the concentration of this catecholamine available at the cellular, tissue, or systemic level (10, 53, 54, 64).

Most research to date has focused on the effects of norepinephrine on splenic macrophage production of tumor necrosis factor-α and transforming growth factor-β with little or no research focusing on the effects of norepinephrine on the production of these cytokines by T cells and murine alveolar macrophage populations (10, 39, 53, 67). It has been reported that although stimulation of macrophage α-adrenergic receptors increases

the production of TNF-α and TGF-β, stimulation of β-adrenergic receptors has the opposite effect, decreasing the secretion of both cytokines (10, 39, 54, 64). These experiments have also illustrated that adrenergic antagonists, when added together with lipopolysaccharide to splenic macrophages, induce a response opposite to that produced by their respective agonist (10, 39). This suggests an important role for norepinephrine in modulating cytokine production (10, 39, 64). As mentioned, very little work has been completed on the effects of norepinephrine on lymphocytes and murine alveolar macrophages, thus these topics will be the focus of this Masters project.

THE MTT COLOURIMETRIC BIOASSAY

Measurement of surviving and/or proliferating mammalian cells is the main requirement of most immunologic assays (68, 69, 70, 71, 72, 73, 74). In the past, this was achieved predominantly through two methods -- the utilization of ^{51}Chromium-labeled protein measurement after cell lysis, or the measurement of radioactive [^{3}H]thymidine or [^{125}I]iododeoxyuridine uptake during cell proliferation (68, 69, 70, 71, 72, 73, 74, 75). Although both biological assays can be partially automated, neither can handle moderately large numbers of samples nor can they be easily performed (68, 69, 70, 71, 72, 73, 74). Furthermore, although viable cells can be measured utilizing the aforementioned techniques as well as by staining (68), these methods are unable to quantify levels or effects of physiological chemicals like cytokines with a high degree of accuracy (68, 69, 70, 71, 72, 73, 74). Mosmann, realizing the shortcomings of these methods, developed a quantitative colourimetric assay for mammalian cell growth using a yellow tetrazolium salt (MTT) (74).

MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) measures only living cells since the conversion of this dye to its final product can only be accomplished by active mitochondrial dehydrogenase enzymes (70, 71, 72, 73, 74, 75). When the tetrazolium ring is cleaved by the active mitochondria of a mammalian cell, the

final product is a soluble blue formazan crystal which can be quantified utilizing a spectrophotometer (70, 71, 72, 73, 74, 75). Because the tetrazolium ring can only be cleaved by active mitochondria, the reaction can therefore only occur in living cells (70, 71, 72, 73, 74, 75). Research has determined that the energy metabolism within a cell reflects the amount of formazan generated, thus, a relationship between cell activity and formazan product exists when utilizing the MTT bioassay; a comparison of cell activation levels can be deduced from the amounts of formazan product produced (70, 71, 72, 73, 74, 75). Although the production of formazan from MTT is restricted by the activation level of the assay cell line, in many situations, such as growth factor assays, optimum cell lines can be pre-selected so as to maximize the formazan production and thus increase the sensitivity of the assay (70, 71, 72, 73, 74, 75).

The cleavage of the MTT ring has various properties that are desirable for assaying cell survival and proliferation (69, 70, 71, 72, 73, 74, 75). Not only is MTT cleaved by all living, metabolically active cells, but it cannot be converted by erythrocytes (70, 72, 73, 74, 75). At present, it appears that all cell types, excluding erythrocytes, are able to reduce the MTT to a blue formazan product allowing for the use of this assay technique in any assay in which living cells must be distinguished from dead cells, or a lack of cells (70, 71, 72, 73, 74, 75). The assay can also be utilized with cytokine sensitive cell lines to quantify the levels of specific cytokines in culture supernatants, for example IL-2 and IL-6 (69, 70, 71, 72, 73, 75).

The use of the MTT colourimetric bioassay to measure cell growth and survival, performed in microtitre plates in conjunction with multichannel pipettors and an automatic scanning spectrophotometer offers many advantages over the assay techniques of old (69, 70, 71, 72, 73, 74, 75). The MTT assay is relatively inexpensive, easily performed, can process a vast quantity of samples in a limited time period and is capable of precise quantification (69, 70, 71, 72, 73, 74, 75). It is also much safer to perform than the other conventional assays because there is no requirement for radioactive compounds (69, 70,

71, 72, 73, 74, 75). At present, an MTT assay to measure the effects of TNF-α has been briefly outlined, however, no literature has defined a protocol for the preparation of an MTT bioassay to measure the effects of TGF-β on cell proliferation (70, 71, 72, 74, 75).

THE OBJECTIVES

The objectives of this research project were numerous. One of the fundamental objectives was the development and calibration of two functional, non-radioactive bioassays which utilize the MTT colourimetric assay technique rather than the radioactive bioassay to quantify TGF-β and TNF-α. Subsequently, the working assays were applied to test the hypothesis that norepinephrine is able to modulate the production of TGF-β and TNF-α by T lymphocyte and alveolar macrophage populations. The models to test these hypotheses utilized transformed T lymphocyte cell lines, murine splenocytes and murine alveolar macrophages.

The overall focus of the experimental work involved in this Masters thesis examined the complex yet intricate relationship between the immune system and the nervous system. The importance of this research is imperative in understanding the contribution of TNF-α and TGF-β to the development and progression of such diseases as idiopathic pulmonary fibrosis and various forms of cancer.

CHAPTER II:

THE MODULATION OF TUMOR NECROSIS FACTOR-α BY NOREPINEPHRINE IN TRANSFORMED AND PRIMARY CELL CULTURES

Evidence of nervous system involvement in immune system function and regulation has been documented and has furthermore been shown to play important roles in many diseases like Idiopathic Pulmonary Fibrosis and cancer. This chapter investigates the modulation of tumor necrosis factor-α (TNF-α) by norepinephrine in four cell populations. Both transformed and primary cultures were stimulated with adrenergic agonist and antagonist drugs, including norepinephrine, in an effort to determine the effect and adrenoceptor target of norepinephrine in modulating TNF-α production by lymphocytes. In this study, norepinephrine significantly augmented TNF-α production by both the Jurkat and EL-4 cells lines and the non-stimulated murine splenocyte population in a dose dependent manner over a two day time course. This increased production of TNF-α was modulated through both the α- and β-adrenoceptors of the various cell cultures. TNF-α production was significantly inhibited by norepinephrine in the CD3 + PMA stimulated murine splenocyte culture through β-adrenoceptor stimulation, however, a significant increase in TNF-α production was observed when α-adrenoceptor modulation occurred. It is apparent from this research, through the use of the various adrenergic agonist and antagonist treatments, that the effect of norepinephrine on TNF-α production is dependent both on the adrenoceptor target and the dosage of the adrenergic treatment.

INTRODUCTION

An Introduction to Tumor Necrosis Factor-α

Tumor necrosis factor-α (TNF-α) is one of the major cytokines released by T cells, macrophages and monocytes (2, 77, 78, 79, 80, 81). TNF-α is a nonglycosylated, 17 kDa protein whose production is triggered mainly in response to Gram negative bacteria (2, 80, 81, 82). It can function both as a mediator of inflammation or as a growth factor (2, 81, 82). TNF-α can also be produced in

response to bacterial toxins, lipopolysaccharides, tumor cells and viruses -- in fact, research has illustrated that interferon-γ can even enhance TNF-α secretion (2, 26, 77). Interestingly, TNF-α is an integral membrane protein of most T cells and macrophages, allowing for these cells to participate in cytotoxic activities when in contact with antigen targets (2, 26, 80).

Two receptors for TNF-α have recently been defined by researchers (26, 80). These receptors are located on virtually all cell types and a soluble form of these receptors is found in both blood serum and urine (2, 26). Although all receptors specific for TNF-α bind the molecule with high affinity, each receptor type mediates a distinct TNF activity including, cytoxicity, margination, migration, and antiviral action. TNF-α is not only able to participate in immune defense through direct interactions, but it is also able to mediate the production of other cell mediators like interleukin-1 and interleukin-6. As a result, TNF-α can serve as a growth stimulator for fibroblasts as well as play important physiological roles in regulating the growth of normal cells (2, 26). At present, the physiological role of the soluble TNF receptor is not well characterized, although it is expected that the soluble receptor out-competes the cell surface receptor and thus serves to inhibit the biological activity of TNF (83).

TNF-α is a bona fide pro-inflammatory cytokine involved in many autoimmune diseases including multiple sclerosis and rheumatoid arthritis (2, 77, 80, 82, 84, 85). In addition TNF-α is an important mediator of endotoxic shock and has been implicated in multiple organ failure due to Gram-negative sepsis (2, 80, 84, 85). TNF-α has also been shown to have a role in diseases of the respiratory system, like idiopathic pulmonary fibrosis, chronic lung disease of the infant, tuberculosis and sarcoidosis (2, 42). Patients with these respiratory diseases have been shown to not only have a marked increase in TNF-α levels, but have also a more severe disease progression, often culminating in death (2, 42). TNF-α is also being studied as a major participant in the cachexia of patients suffering with AIDS and cancer (2, 42).

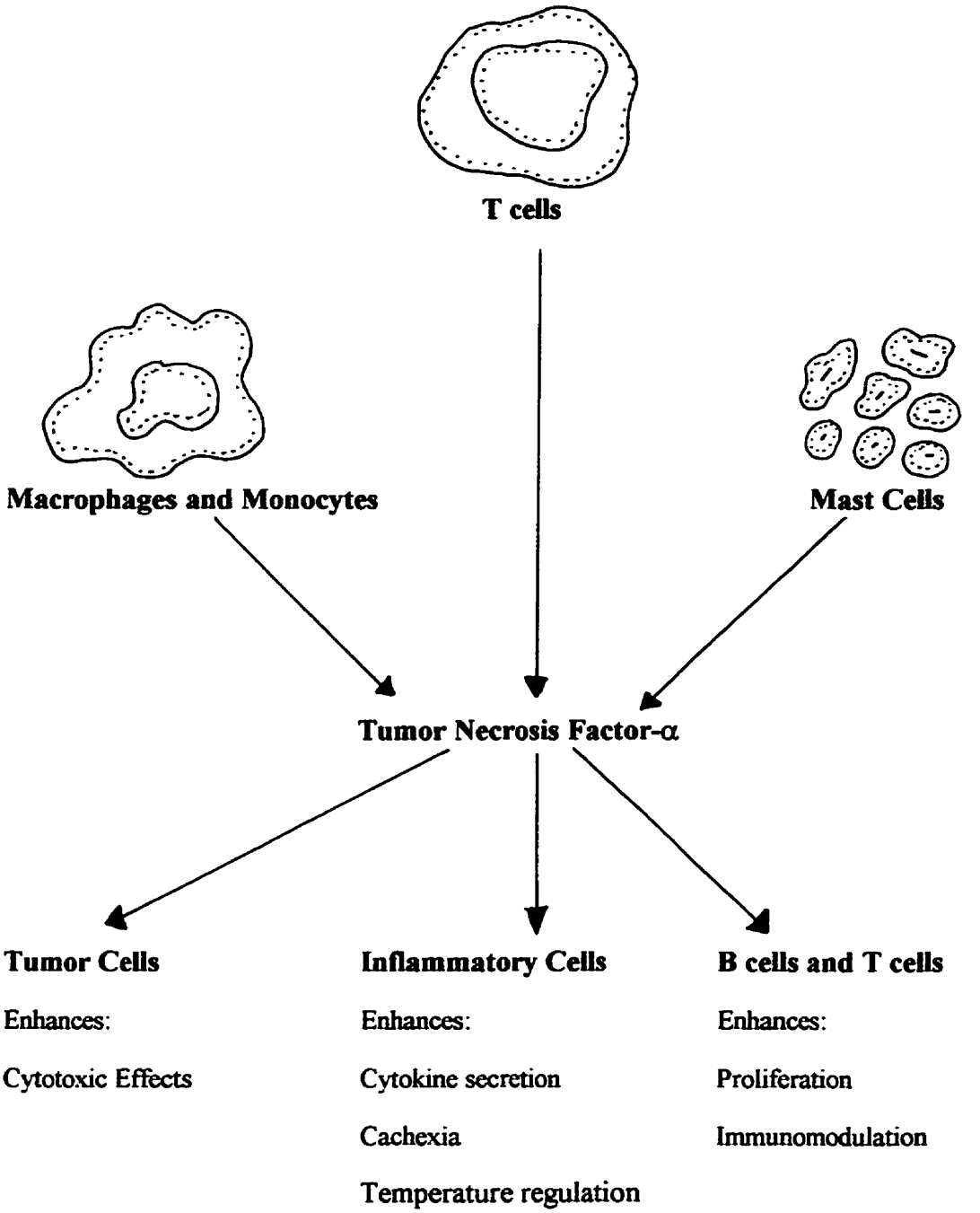


FIGURE 1. The origins and targets of Tumor Necrosis Factor-α (26).

Because TNF-α can participate in a wide variety of immunological effects in many disease and sicknesses, not only is it important to understand the function TNF-α mediates in immune defenses, but it is imperative that the endogenous mechanisms that control the production of this cytokine also be resolved (2, 77, 85).

The T Helper Cell Response and Tumor Necrosis Factor-α

The interaction of resting T cells with antigen and cytokines results in their activation, proliferation and differentiation (28, 35, 86). This activation often results in the release of the T cells' cytokines which can affect both the growth and differentiation of T cells themselves, as well as B cells and other cells involved in an immune response (28, 86, 87, 88). T helper cells are able to secrete two distinct patterns of cytokines that result in very different immune responses (26, 28, 35, 86). CD4+ effector Th cells have been divided into two distinct subsets based on these cytokines -- Th1 cells are recognized as T cells which produce IL-2 and IFN-γ while Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13; both subsets of T cells can secrete a number of cytokines including IL-3, GM-CSF and TNF (28, 35, 86, 87, 88, 89). The differential cytokine production by these effector T helper cells essentially regulates the nature of most immune responses with Th1 cells functioning primarily in cell-mediated immune responses while Th2 cells function primarily in humoral immune responses (28, 86, 89). Although Th1 and Th2 cell responses are necessary for the maintenance of immune homeostasis, often the dominance of one response over another plays an important role in either the progression or clearance of many disease states (28, 86, 89).

Norepinephrine and Tumor Necrosis Factor-α -- A Relationship?

One potential physiological mechanism that may influence cytokine production by CD4+ T helper cells involves the release of norepinephrine by the sympathetic nervous system (50, 89). Published data have illustrated a role for norepinephrine in immune regulation and modulation (21, 50, 89). Norepinephrine-containing nerve terminals enter many lymphoid organs, and therefore T cells which reside within the lymphoid sheath entertain contact with this neurotransmitter. These T cells have been shown experimentally to preferentially express the β_2-adrenergic receptor (50, 89). It has also been discovered that following immunization, norepinephrine is released from these nerve terminals to stimulate the β_2-adrenoceptor on lymphocytes (21, 50, 89). This stimulation of the β-adrenoceptor causes an increase in the intracellular concentration of cAMP (89, 112).

While previous research has examined the effects of norepinephrine on the production of IL-2 and interferon-γ by T helper cells, research has yet to be performed on the involvement of norepinephrine in the production of TNF-α by T lymphocytes (50, 89. 82). To date, it has been reported that exposure of Th1 clones to β_2-adrenergic agonists before activation inhibits production of IL-2 and IFN-γ, but does not affect the production of the Th2 cytokines -- IL-4 or IL-10 (50, 89). The mechanism responsible for these effects is thought to include a differential level of β_2-adrenoceptor expression on resting effector Th cell subsets, as opposed to a differential level of responsiveness of the subsets to cAMP (50, 89). It has been experimentally concluded that while Th1 cells express a detectable level of the β_2-adrenoceptor, no Th1 cells do (89). Moreover, the activation of helper T cells induces a change in the number and type of surface molecules and therefore it has been proposed that a similar activation-induced change may occur in the concentration of β_2-adrenoceptor expression by this T cell subset (89).

Although the influence of the β-adrenoceptor has been studied in great detail, the involvement of α-adrenoceptor activation on the modulation of the immune response of helper T cells remains unclear (90). Furthermore, although much research has been performed on the effects of norepinephrine on macrophage produced TNF-α, work has yet to be published on the effects of norepinephrine regarding the modulation of TNF-α production by T cells. The experiments and data displayed in this chapter will examine the modulation of TNF-α production by various T lymphocyte cultures.

An MTT Assay for Tumor Necrosis Factor-α

Bioassaying tumor necrosis factor-α in biological fluid is widespread and fundamentally relies on a stable cell line sensitive to TNF-α action (76, 91). The bioassaying of TNF-α is particularly desirable, both because of its involvement in various pathological and inflammatory responses and also because of the many disease conditions where total bioactivity is of concern due to cytokines, including, but not limited to TNF-α (76, 91). Several systems for a TNF-α bioassay have appeared and been reviewed in the literature, but often the stability of the cell lines is limited -- four months is often the maximum length for which these systems have been found reliable (76, 91). Presently, the accepted bioassay method for TNF-α utilizes the WEHI-13VAR cell line which was obtained after serial passages of the WEHI 164 clone 13, the original bioassay cell line for TNF-α (76, 91, 92). Current results have indicated that the WEHI-13VAR cell line, when used in conjunction with actinomycin, serves as a stable and sensitive cell line for TNF-α detection and quantification. Although the role of actinomycin in provoking the sensitivity of these cells towards TNF-α is not understood, it is believed that actinomycin may stabilize the reaction of the cells to the cytotoxic action of TNF-α and thus may serve to increase the sensitivity of the classical assays (76, 91).

The WEHI-13VAR assay for TNF-α is based on the cytotoxic effects of TNF-α (83). The established protocol for the WEHI-13VAR bioassay currently relies on the use of MTT dye which is capable of measuring only living cells since the conversion of this yellow dye to its final product can only be accomplished by active mitochondrial dehydrogenase enzymes (74). When combined with the MTT method, the TNF-α content of a sample can be determined by comparing the conversion of the blue formazan product of the sample to a standard curve utilizing rTNF-α. Thus, the amount of formazan product is directly related to the cell viability after TNF-α sample addition (74, 91). Finally, although this bioassay can be used to measure TNF, it is unable to differentiate between TNF-α and TNF-β and therefore specific monoclonal antibodies for TNF-α must be added to duplicate samples in order to verify that the cytokine having the effect is in fact TNF-α (76, 91).

MATERIALS AND METHODS

MATERIALS

A complete listing of the source and all types of media, solutions, specific chemicals and equipment used in this thesis, outlined in the methods section is attached as Appendix A.

METHODS

Transformed murine EL-4 T cells and human Jurkat lymphoma cell collections. Both EL-4 (ATCC TIB39) and Jurkat (ATCC CRL8163) cell lines were continuously cultured in individual 25 cm^2 flasks (Fisher Scientific) in RPMI-1640 (Gibco Laboratories) supplemented with 10% FBS (Sigma Chemical Co.), 1.0×10^{-3} M sodium pyruvate (Sigma Chemical Co.), 2.0×10^{-3} M L-glutamine (Sigma Chemical Co.), and 5 mg/mL gentamycin sulfate (Sigma Chemical Co.). Each

experimental flask and plate prepared for supernatant collection consisted of only one cell line, and each experiment was tested for only one cytokine -- tumor necrosis factor-α.

Cells were removed from their respective culture flasks and centrifuged for ten minutes at 1000 RPM at 4°C. The supernatant was decanted and the cell pellet was resuspended at a final concentration of 1.0×10^6 cells/mL in RPMI-1640 supplemented with 10% FBS, 1.0×10^{-3} M sodium pyruvate, 2.0×10^{-3} M L-glutamine, and 5 mg/mL gentamycin sulfate. Subsequently, 495 μL of the cell solution was added to each well of a 24-well tissue culture treated microtitre plate (Fisher Scientific). The final cell concentration of each well was thus approximately 5.0×10^5 cells/well. Cell viability, determined by exclusion of trypan blue, was >95% in all experiments.

In vitro effects of catecholamines on transformed murine EL-4 T cells and human Jurkat lymphoma cell collections. Transformed T cells obtained from cell culture methods, as previously described, were cultured in the presence of norepinephrine (Sigma Chemical Co.), terbutaline (Sigma Chemical Co.), UK14304 (A. McNichol, University of Manitoba), methoxamine (Sigma Chemical Co.), phentolamine (Sigma Chemical Co.), propranolol (Sigma Chemical Co.), media (Gibco Laboratories) a 1:1 solution of norepinephrine-propranolol, or a 1:1 norepinephrine-phentolamine solution at one of three concentrations (10^{-4} M, 10^{-6}M and 10^{-8} M) for 24 hrs and 48 hrs. At each of the time course points, the conditioned media was collected as described below. The pharmacologic reagents were prepared at 10-fold higher concentrations in RPMI-1640 supplemented with 10% FBS, 1.0×10^{-3} M sodium pyruvate, 2.0×10^{-3} M L-glutamine, and 5 mg/mL gentamycin sulfate and added to the 24-well tissue culture plates prepared with lymphocytes at a volume of 5 μL/well.

After aliquoting the cells and the pharmacologic reagents, the plates were allowed to incubate for a two day time course at 37°C, 95% CO_2. Samples of conditioned media were collected following 24 and 48 hrs of incubation, and stored at -80°C until ready for TNF-α quantification (93). These experiments were performed on each cell line a minimum of six times.

Animals. Male, CR1:CD-1 (ICR)Br specific pathogen-free mice (Charles River) were used between six weeks and six months of age. Mice were housed in groups of five on woodchip bedding and were provided autoclaved food and water ad libitum. The requirements of the University's Animal Care and Ethics Committee were met for all experimental procedures. A minimum of six mice were sampled over the course of each of the murine splenocyte experiments.

Murine Spleen Harvest and Culture. Mice were individually euthanized with carbon dioxide and the spleen was removed aseptically and placed in sterile RPMI-1640 supplemented with 10% FBS. Single cell suspensions were recovered from the spleen by disaggregating them on a stainless steel, sterile mesh and adding RPMI-1640 supplemented with 10% FBS. The cell suspension was washed by centrifugation for 10 minutes at 1000 RPM at 4°C and the pellet was subsequently resuspended in a small volume of RPMI-1640 supplemented with 10% FBS prior to the lysis of erythrocytes with 0.017 M tris-ammonium chloride buffer (Sigma Chemical Co.). Cells were again washed after the lysis of red blood cells, as previously described, and resuspended at a final concentration of 1.0×10^6 cells/mL in RPMI-1640 supplemented with 10% FBS, 1.0×10^{-3} M sodium pyruvate, 2.0×10^{-3} M L-glutamine, and 5 mg/mL gentamycin sulfate.

Preparation of Monoclonal Antibody. Hybridoma 145-2C11 (ATCC CRL 1975) was cultured in RPMI-1640 supplemented with 10% FBS, 1.0 x 10^{-3} M sodium pyruvate, 2.0 X 10^{-3} M L-glutamine, and 5 mg/mL gentamycin sulfate. The supernatant was collected regularly and stored at 4°C; all hybridoma cells were removed by centrifugation prior to storage. Saturated ammonium sulphate was added to the antibody solution until 40-45% saturation was achieved. The precipitate was allowed to form overnight at 4°C. The 40-45% saturated ammonium sulphate slurry was resuspended and subsequently centrifuged at 10000 xG for 5 minutes. The precipitate was purified against 200 volumes of 0.15 M NaCl, changing the fluid 3 times at 4°C. The mAb was sterilized by filtration through 0.22 μm filters (Nalgene) and stored for future use, at 4°C.

Activation of murine T lymphocyte population via CD3 + PMA. The optimal concentration of 145-2C11 monoclonal antibody has previously been defined as 33 μg/mL when titrated using murine splenic lymphocytes at 4 months of age (94). Preparation of experimental plates (Fisher Scientific) with 145-2C11 monoclonal antibody has also been previously described (94). Briefly, 96-well tissue culture treated plates were coated with 145-2C11 monoclonal antibody at 33 μg/mL in sterile PBS at 100 μL/well. To achieve coating, the 145-2C11 monoclonal antibody was incubated in plates overnight at 4°C. Unbound monoclonal antibody was removed before experimentation by washing the plates three times with RPMI-1640 supplemented with 5% FBS. Murine spleen cells, prepared as previously described, were then added to the mAb-coated plates at 1.0 x 10^6 cells/mL in RPMI-1640 supplemented with 10% FBS, 1.0 x 10^{-3} M sodium pyruvate, 2.0 X 10^{-3} M L-glutamine, 5 mg/mL gentamycin sulfate and 10 ng/mL phorbol myristate acetate (PMA) (Sigma Chemical Co.) using 100 μL cells/well. A set of plates, which served as control plates were also prepared. These plates contained only PBS for the

overnight incubation and subsequently had only murine splenocytes added to them in combination with an adrenergic reagent, as described below.

In vitro effects of catecholamines on CD3 + PMA activated and non-activated murine splenic cells. Splenic cells obtained from spleen harvest procedures as previously described (95) were cultured in the presence or absence of CD3 mAb and PMA plus one of norepinephrine, terbutaline, UK14304, methoxamine, phentolamine, propranolol, a 1:1 solution of norepinephrine-propranolol, or a 1:1 norepinephrine-phentolamine solution at three concentrations (10^{-4}, 10^{-6} and 10^{-8}) for 24 hrs and 48 hrs. From each mouse spleen, the total cell volume was divided so that one series of samples was run without stimulation (no PMA or CD3), and one set was run with stimulation (both PMA and CD3 were present). At each of the two time course points, the conditioned media was collected as described below. The pharmacologic reagents were prepared at 10-fold higher concentrations in RPMI-1640 supplemented with 10% FBS, 1.0 x 10^{-3} M sodium pyruvate, 2.0 X 10^{-3} M L-glutamine, and 5 mg/mL gentamycin sulfate and added to the murine spleen cell suspensions immediately before their addition to the CD3-coated tissue culture plates.

After aliquoting the cells and the pharmacologic reagents, the plates were allowed to incubate for a two day time course at 37°C, 95% CO_2. Samples of conditioned media were collected following 24 and 48 hrs of incubation and stored at -80°C until ready for TNF-α quantification. As mentioned, a minimum of six mice were sampled for the unstimulated splenocyte experiments, and a further minimum of six mice were utilized in the CD3 + PMA stimulated murine splenocyte experiments.

TNF-α assay. A bioassay sensitive for TNF-α activity was used to determine the concentration of TNF-α in the collected conditioned media. The WEHI-13VAR cytotoxicity assay, based on a previously reported procedure with some modifications

was utilized (76, 91, 96), and TNF-α content of the supernatants was measured and expressed as ng/mL. WEHI-13VAR cells were used to assay the previously collected samples for TNF-α (76, 91). Briefly, subconfluent WEHI-13VAR cells were washed, trypsinized and washed again with RPMI-1640 supplemented with 5% FBS. The collected cell solution was centrifuged and the cell pellet resuspended in RPMI-1640 supplemented with 10% FBS, 1.0×10^{-3} M sodium pyruvate, 2.0×10^{-3} M L-glutamine, 5 mg/mL gentamycin sulfate and 4.5 mg/mL glucose and plated at a concentration of 1.5×10^4 cells/100 μL in a 96 well microtitre plate. The cells were allowed to adhere overnight, for approximately 15 hrs. Post-incubation, the medium was removed utilizing a multichannel pipettor, and 50 μL of the collected conditioned media samples was added in the presence or absence of anti-TNF-α antibodies (Peprotech). To this, 50 μL of a 0.2 μg/mL Actinomycin D (Sigma Chemical Co.) solution was added. Each of the wells contained a final total volume of 100 μL (76, 91, 96).

After 18 hrs of further incubation at 37°C, 95% CO_2, 25 μL of a 5 mg/mL MTT (Sigma Chemical Co.) solution was added to each well and the plate was allowed to further incubate at 37°C, 95% CO_2 for six hours (74). The contents of the wells were then carefully removed by gently inverting and blotting the microtitre plate (74). Finally after 100 μL/well of a stop solution of acidified SDS was added to each well, the plate was vigorously shaken for 25 minutes, following which the absorbance was read in a 750 Cambridge plate reader at 590 nm (74). The activity of the TNF-α in the samples was calculated using an rTNF-α (Peprotech) standard prepared at the same time as the experimental samples (76, 91).

Prior to the use of the WEHI-13VAR assay for quantifying TNF-α, the assay was calibrated for use. The cell number which would give maximal OD readings at 590 nm was selected, and a standard of rTNF-α was defined to be utilized each time

the assay was run. This pre-defined standard from 0 ng/mL rTNF-α to 250 ng/mL was used to verify the sensitivity of the assay throughout the experiments (76, 91).

Also prior to the use of the WEHI-13VAR assay for quantifying TNF-α, the adrenergic drug treatments were added to the WEHI-13VAR cells after their first incubation to determine whether or not the adrenergic drug treatments had an effect on the WEHI-13VAR cells. Post incubation of the cells after the initial plating, the medium was removed utilizing a multichannel pipettor and 50 μL of each adrenergic treatment at the maximum experimental concentration (10^{-4} M) was added. Subsequently, 50 μL of a 0.2 μg/mL Actinomycin D solution was added and following the second incubation, the plates were read and a comparison of the optical density readings were made to WEHI-13VAR cells which did not receive an adrenergic drug. It is imperative that the WEHI-13VAR cells be sensitive only to TNF-α content and not to the adrenergic drug treatment.

When the assay was utilized to quantify TNF-α in collected supernatant samples, the samples were dividied so that triplicate repeats could be performed. After obtaining all data, the mean of the data was determined as well as a calculation of the standard deviation of the mean.

TNF-α Statistical Analysis. The data are expressed as total TNF-α content (ng/mL). Values are compared using the Minitab Statistical Software for ANOVA. All values are reported as the mean ± the standard deviation and analyzed by Tukey's pairwise comparison test (n=6). An alpha error of 0.05 was used to test for statistical significance.

RESULTS

The Tumor Necrosis Factor-α MTT Bioassay and Calibration

Effects of cell number on formazan production by WEHI-13VAR murine fibrosarcoma cells in combination with a rTNF-α standard. To allow for determination of the effect of cell number on MTT conversion to blue formazan, five concentrations of WEHI-13VAR cells were selected for pre-testing with a shortened rTNF-α standard. The cell number ranged from 1.0×10^4 cells/mL to 1.0×10^6 cells/mL. A short standard of rTNF-α was prepared ranging from 0 ng/mL to 50 ng/mL and was added to the pre-incubated cells. After incubation, the plates were read and a comparison of the optical density readings was made. It is important for there to be a wide range in OD readings between the maximal rTNF-α concentration and the 0 ng/mL rTNF-α concentration to provide a high level of sensitivity for the assay. This wide range was present with the cell concentration of 1.5×10^5 cells/mL, therefore, a cell concentration of 1.5×10^5 cells/mL was chosen for subsequent TNF-α assay procedures (Figure 2).

Effects of adrenergic agonist and antagonist treatments on the WEHI-13VAR cell line. To allow for determination of the effect of the adrenergic agonist and antagonist treatments on the WEHI-13VAR cell line, all adrenergic drug treatments were co-incubated at their maximum concentration (10^{-4} M) with the WEHI-13VAR cell line at the same point where sample addition would occur in the TNF-α assay procedure. The results from this experiment are illustrated in Figure 3. The results display that none of the adrenergic treatments had an effect on the WEHI-13VAR cell line as each of the treatments had a similar optical density value to WEHI-13VAR cells cultured in the absence of an adrenergic treatment.

FIGURE 2. A TNF-α standard curve utilizing WEHI-13VAR cells at a concentration of 1.5×10^5 cells/mL. A short standard from 0 ng/mL to 50 ng/mL rTNF-α was prepared and sampled with five concentrations of WEHI-13VAR cells. The varying concentrations of WEHI-13VAR cells displayed different optical density readings over the same rTNF-α standard. The samples containing the WEHI-13VAR at a concentration of 1.5×10^5 cells/mL gave the greatest difference in optical density readings over the 0 ng/mL to the 50 ng/mL rTNF-α concentrations. The rTNF-α standard curve obtained for this cell concentration utilizing the defined standard is displayed. Each point on the graph represents the mean of a triplicate and is shown with error bars noting the standard deviation of the mean.

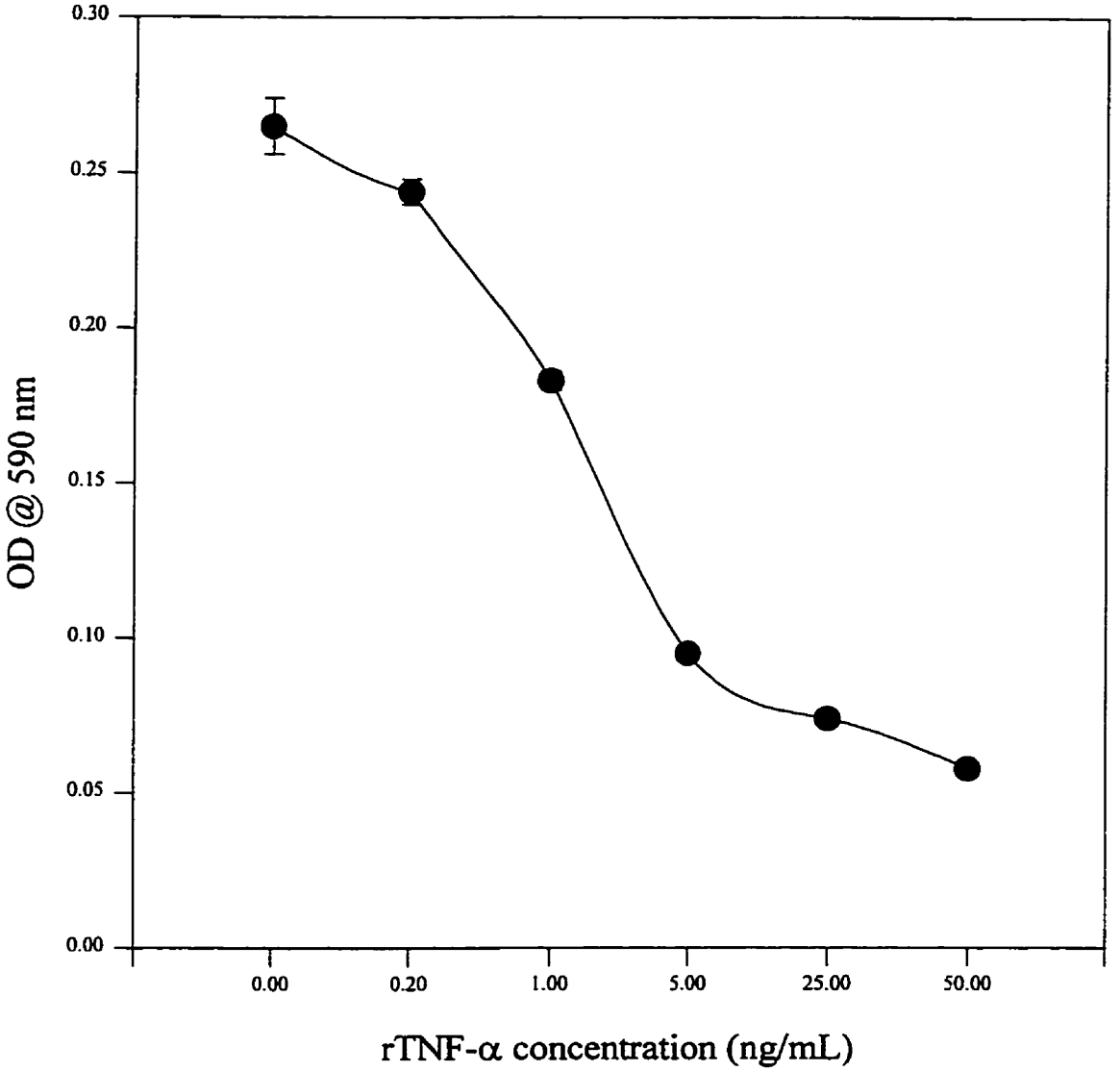
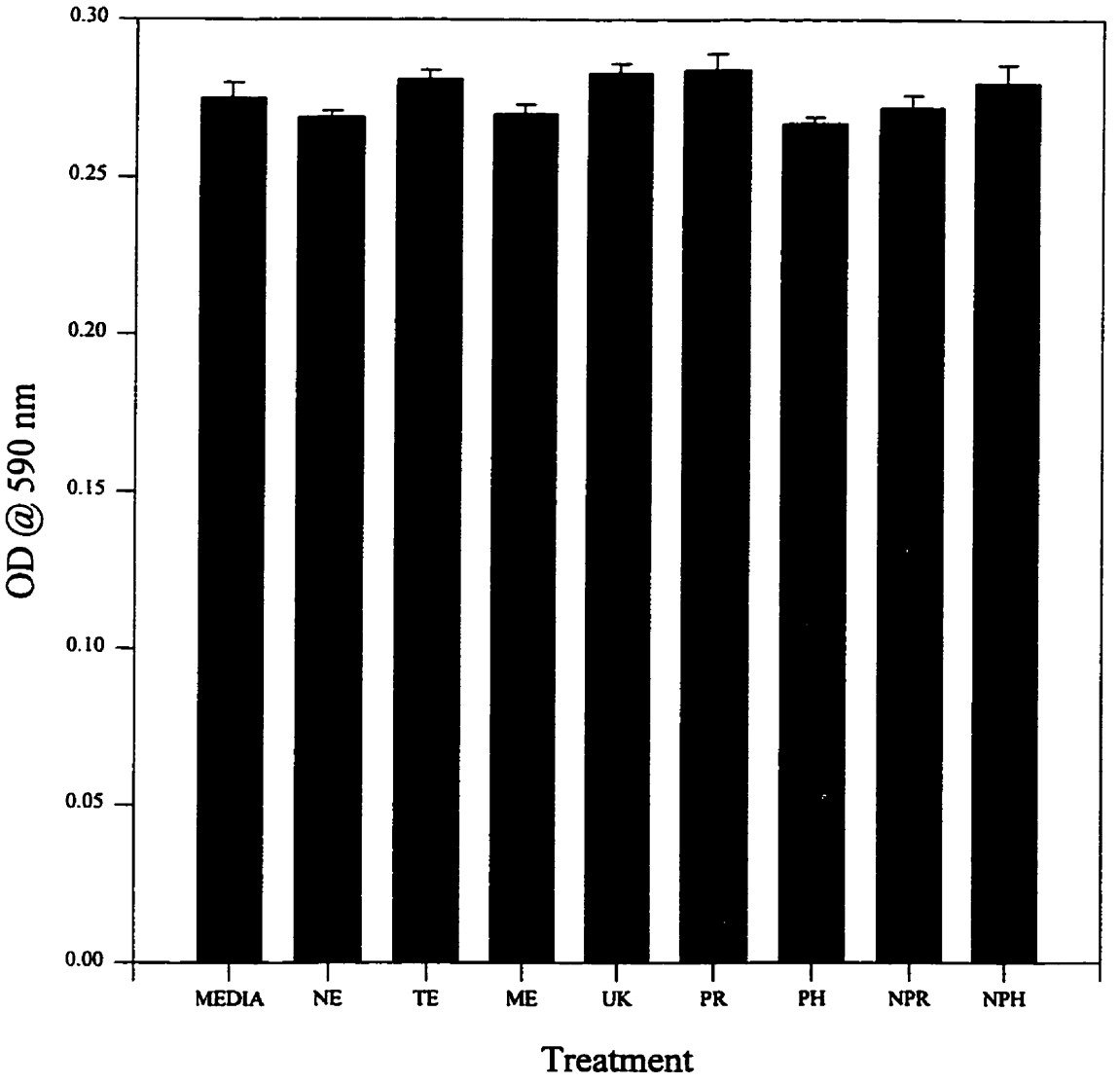

0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD @ 590 nm
0.00
0.20
1.00
5.00
25.00
50.00
rTNF-α concentration (ng/mL)

FIGURE 3. Effects of adrenergic agonist and antagonist treatments on the WEHI-13VAR cell line. The adrenergic agonist and antagonist treatments were added to the WEHI-13VAR cell line after the first overnight incubation to test the effects of the various adrenergic treatments on the WEHI-13VAR cells. This addition of adrenergic treatments took place at the same time point as the addition of conditioned media samples would occur in the TNF-α assay procedure. The different adrenergic treatments did not have any significant effect on the optical density readings as compared to WEHI-13VAR cells treated only with media. Each point on the graph represents the mean of a triplicate and is shown with error bars noting the standard deviation of the mean.


0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD @ 590 nm
MEDIA
NE
TE
ME
UK
PR
PH
NPR
NPH
Treatment

Adrenergic Agonist and Antagonist Treatments, Jurkat Cells and Tumor Necrosis Factor-α

The production of TNF-α by Jurkat cells in response to adrenergic agonists and antagonists (NE, TE, ME, UK, PR, PH, NPR, NPH and media) was tested over a concentration range of 10^{-4}, 10^{-6}, 10^{-8} M. Samples of conditioned media were collected over a two day time course and assayed for TNF-α. The results of these experiments are displayed in Figure 4 -- 24 hr data is illustrated with solid bars, while the 48 hr data is represented by the hatched bars.

From the obtained experimental results, it is observed that NE can significantly increase the production of TNF-α by Jurkat cells. Since NE can act through both α- and β-adrenoceptors, and because cells of the immune system are known to have both types of these receptors, the effects of the individual adrenoceptor in response to NE were examined. When the β_2-adrenoceptor of the Jurkats was directly stimulated with TE, TNF-α production by the Jurkat cells was significantly increased. Furthermore, when the ability of NE to act through the α-adrenoceptor was blocked with PH (NPH treatment), the production of TNF-α was again significantly augmented through the β-adrenoceptor. These combined results thus suggest that NE can significantly increase TNF-α production through the β-adrenoceptor of the Jurkat cell.

Subsequently, an investigation into the α-adrenoceptor effect of NE was performed utilizing ME and UK, α_1- and α_2-adrenergic agonists, respectively. By directly stimulating the α_1-adrenoceptor with ME, TNF-α production was significantly increased. Likewise, when the α_2-adrenoceptor was stimulated with UK, TNF-α production was also significantly increased. When the Jurkat cells were treated with the NPR treatment, the β-adrenoceptor should have been blocked by PR, and therefore NE would only have access to the α-adrenoceptor. In the results obtained from the NPR adrenergic treatments, TNF-α production was again significantly increased. This group of results, examining the effects of α-adrenoceptor modulation

FIGURE 4. A comparison of mean total TNF-α production over a two day time course by Jurkat cells treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the total TNF-α production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

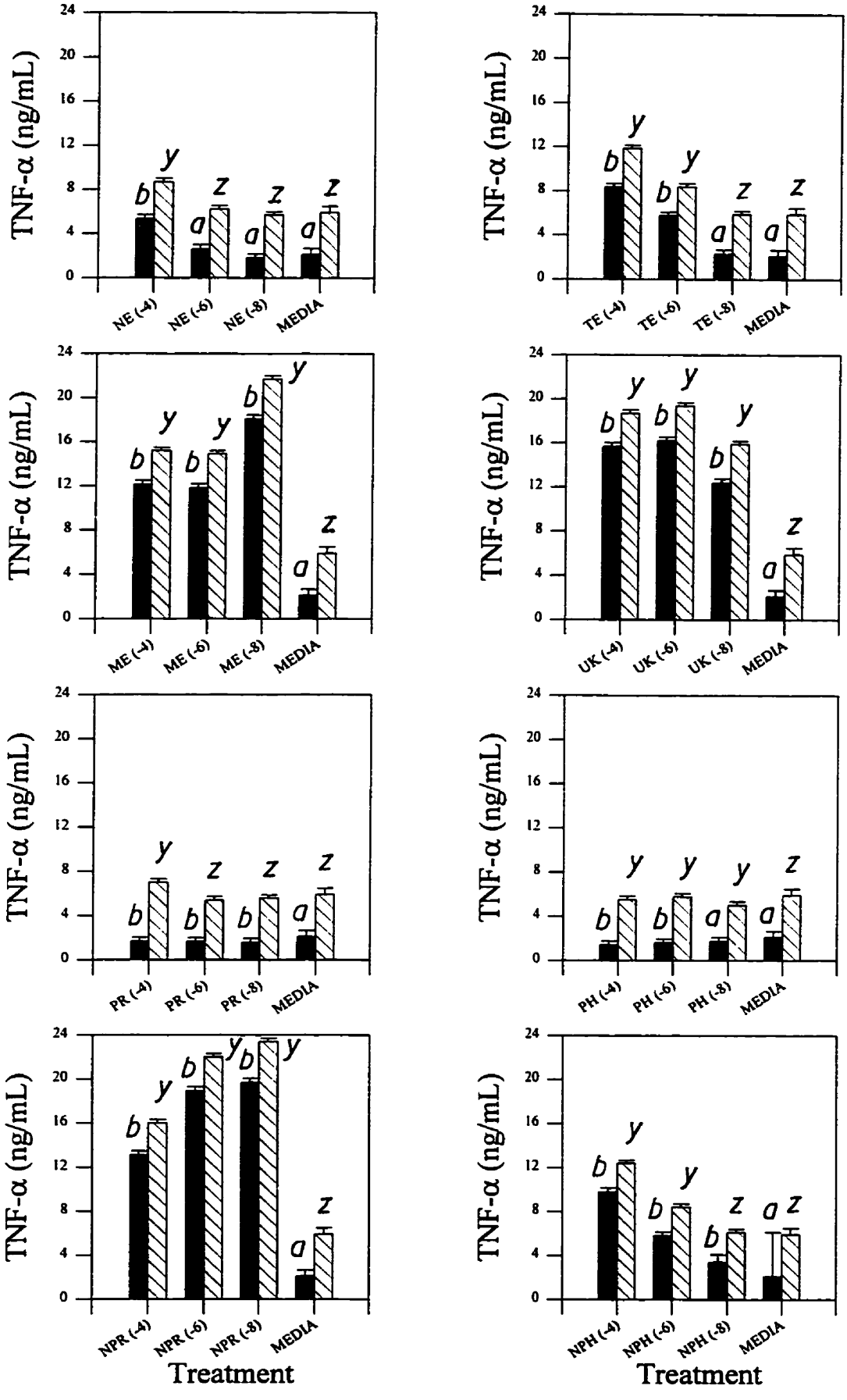
Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	PR	≤0.082	≤0.001
TE	≤0.001	≤0.001	PH	≤0.019	≤0.003
ME	≤0.001	≤0.001	NPR	≤0.001	≤0.001
UK	≤0.001	≤0.001	NPH	≤0.001	≤0.001

Adrenergic Treatment Abbreviations:

NE:	Norepinephrine	PR:	Propranolol
TE:	Terbutaline	PH:	Phentolamine
ME:	Methoxamine	NPR:	Norepinephrine + Propranolol
UK:	UK14304	NPH:	Norepinephrine + Phentolamine

Adrenergic Treatment Concentrations:

(-4):	10(-4) M	(-6):	10(-6) M	(-8):	10(-8) M


TNF-α (ng/mL)
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

by UK, ME and NPR treatments, suggests that NE can significantly increase TNF-α production through the α-adrenoceptor of the Jurkat cell.

In summary, these results suggest that NE can act through either the α- or β-adrenoceptor, with some preference perhaps for the β-adrenoceptor since the effects of NE were similar to those observed when the Jurkats were stimulated with TE. The effect of NE also appears to be dose dependent in regards to β-adrenoceptor modulation of TNF-α production, with higher concentrations of NE resulting in greater quantities of TNF-α being produced. It is also apparent that NE can act through both subsets of α-adrenoceptors to modulate increased TNF-α, however this effect does not appear to depend solely on the concentration of the α-adrenergic treatment.

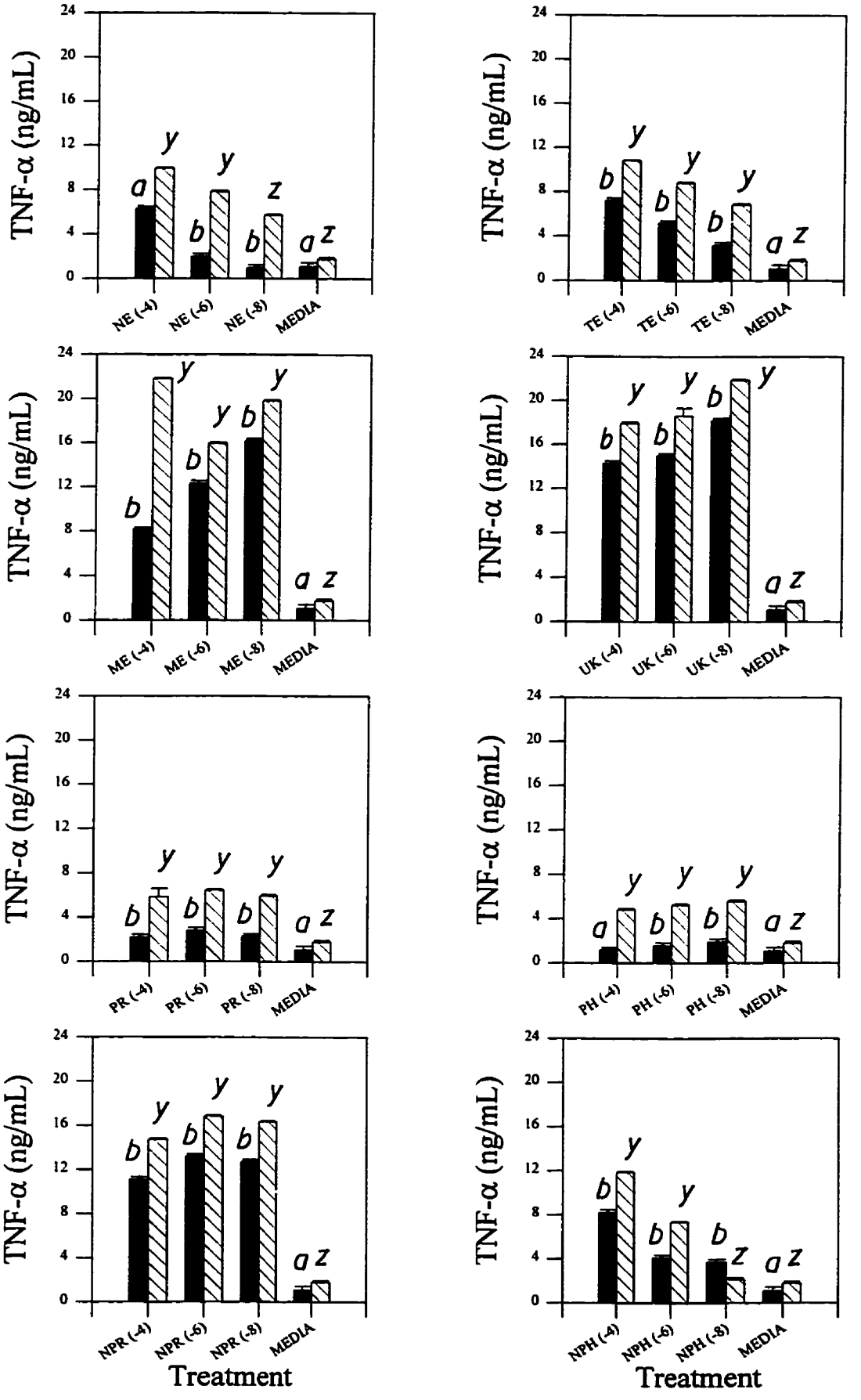
Evaluating the above data for total TNF-α production by Jurkat cells over the two day time period using Tukey's pairwise comparison illustrates that there are significant differences between the media control treatment and some adrenergic treatments, but not others. The addition of anti-TNF-α antibodies to duplicate samples of conditioned media demonstrated that the effects illustrated throughout the experiments were due to TNF-α and thus proved that the WEHI-13VAR assay was able to measure the quantities of TNF-α secreted by the Jurkat cells.

Adrenergic Agonist and Antagonist Treatments, EL-4 Cells and Tumor Necrosis Factor-α

The production of TNF-α by EL-4 cells in response to the adrenergic agonists and antagonists was also tested over the concentration range of 10^{-4}, 10^{-6}, 10^{-8} M. Samples of conditioned media were collected over a two day time course and assayed for TNF-α. The results of these experiments are displayed in Figure 5 -- 24 hr data is illustrated with solid bars, while the 48 hr data is represented by the hatched bars.

FIGURE 5. A comparison of mean total TNF-α production over a two day time course by EL-4 cells treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the total TNF-α production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	PR	≤0.001	≤0.001
TE	≤0.001	≤0.001	PH	≤0.001	≤0.001
ME	≤0.001	≤0.001	NPR	≤0.001	≤0.001
UK	≤0.001	≤0.001	NPH	≤0.001	≤0.001


TNF-α (ng/mL)
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

It is illustrated from preliminary observations that NE can signficantly increase the production of TNF-α by EL-4 cells. Again, because of NE's dual ability to act through both α- and β-adrenoceptors, it was necessary to determine the effects of the individual adrenoceptors in response to NE. When utilizing TE to stimulate the β_2-adrenoceptor of the EL-4s, TNF-α production was significantly increased. Subsequently, when the α-adrenoceptor was blocked by PH in the NPH treatment, the production of TNF-α was again observed to be significant through the β-adrenoceptor. This combined set of results suggest that NE, when acting through the β-adrenoceptor of the EL-4 cell line, can significantly increase TNF-α production.

Further investigations regarding the α-adrenoceptor effects of NE on EL-4 production of TNF-α were performed utilizing ME and UK. When the α_1-adrenoceptor was stimulated with ME, TNF-α production was significantly increased. In a similar manner, when UK was utilized to stimulate the α_2-adrenoceptor, TNF-α production was also significantly increased. When the ability of NE to access the β-adrenoceptor of the EL-4 cell was eliminated through the use of PR (the NPR treatment), NE continued to significantly increase TNF-α production. These combined results, examining the effects of α-adrenoceptor modulation, suggest that NE can increase the production of TNF-α significantly through the α-adrenoceptor of the EL-4 cell.

Finally, the effect of each of the adrenergic antagonists alone on the production of TNF-α by EL-4 cells was determined. From these results, it appears that both PR and PH treatments were individually able to significantly increase TNF-α production by the EL-4 cell line.

In conclusion, these results suggest that NE can act through either the α- or β-adrenoceptor of the EL-4 to modulate increased production of TNF-α. It appears that there again may be some preference by NE for the β-adrenoceptor since the effects of NE were similar to those observed when the EL-4s were stimulated with TE. The

effect of NE on β-adrenoceptor modulation of TNF-α production also appears to be dose dependent with maximum production of TNF-α occurring at the maximum concentration of NE. It is also apparent when examining the experimental results that NE can modulate increased TNF-α production through the α-adrenoceptor, however this effect does not necessarily relate to the concentration of the α-adrenergic drug.

Evaluating the above data for total TNF-α production by EL-4 cells over the two day time period using Tukey's pairwise comparison illustrates that there are significant differences between the media control treatment and some adrenergic treatments, but not others. The addition of anti-TNF-α antibodies to duplicate samples of conditioned media demonstrated that the effects illustrated throughout the experiments were due to TNF-α and thus proved that the WEHI-13VAR assay was able to measure the quantities of TNF-α secreted by the EL-4 cells.

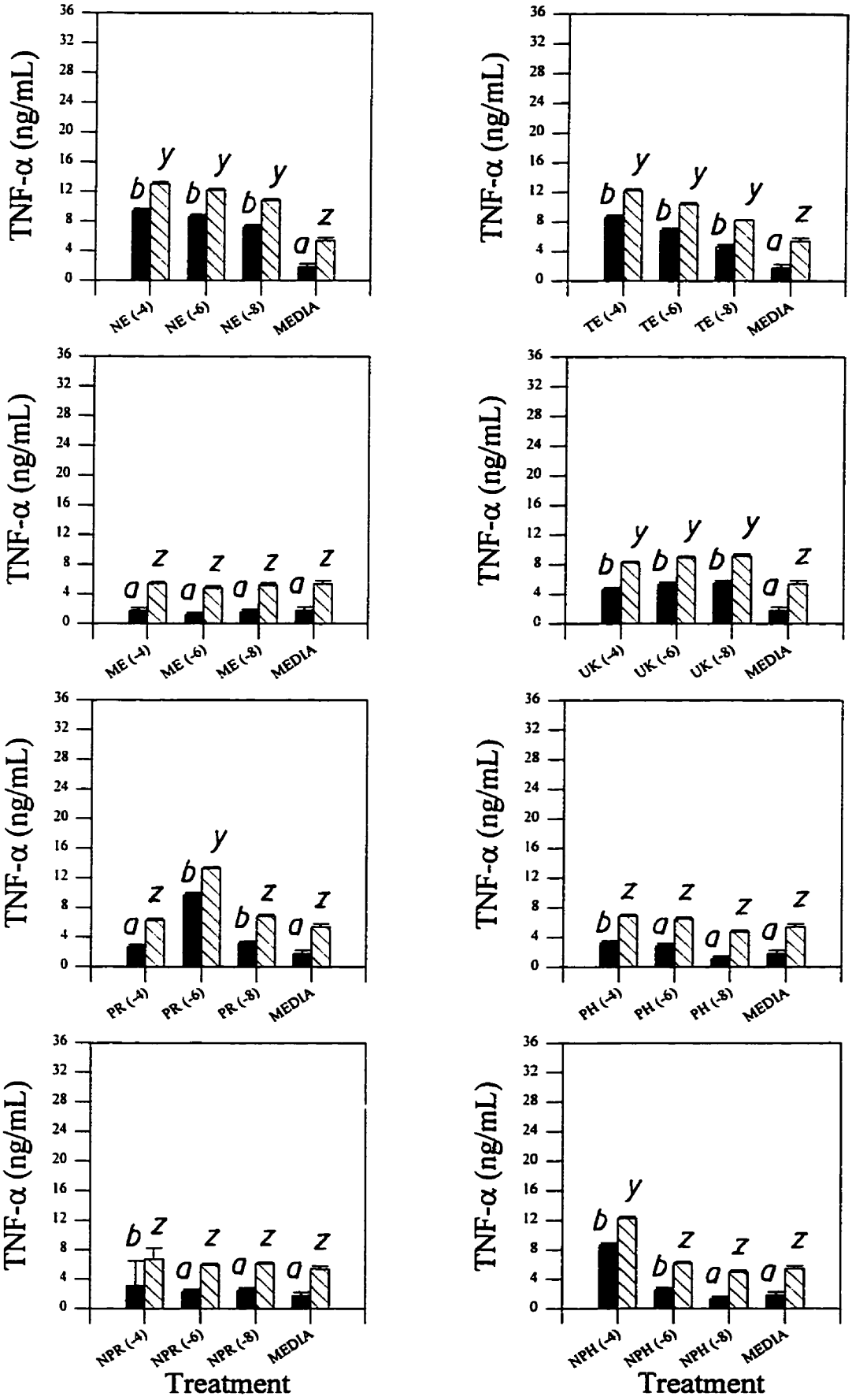
Adrenergic Agonist and Antagonist Treatments, Non-Stimulated Murine Splenocytes and Tumor Necrosis Factor-α

The production of TNF-α by non-stimulated murine splenocytes in response to adrenergic agonist and antagonist treatments was also tested over the concentration range of 10^{-4}, 10^{-6}, 10^{-8} M. Samples of the conditioned media were collected over a two day time course and assayed for TNF-α. The results of these experiments are displayed in Figure 6 -- 24 hr data is illustrated with solid bars, while the 48 hr data is represented by the hatched bars.

Many of the treatments of adrenergic agonists and antagonists had statistically significant effects on the production of TNF-α. It is illustrated that NE had a significant effect on the production of TNF-α by non-stimulated murine splenocyte populations, and the results display that the individual α- and β-adrenergic treatments had the same α- and β-adrenoceptor specific effects previously observed in the transformed cell lines -- with stimulation of either adrenoceptor resulting in an increase

FIGURE 6. A comparison of mean total TNF-α production over a two day time course by non-stimulated murine splenocytes treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the total TNF-α production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	PR	≤0.001	≤0.001
TE	≤0.001	≤0.001	PH	≤0.001	≤0.001
ME	≤0.007	≤0.001	NPR	≤0.001	≤0.001
UK	≤0.001	≤0.001	NPH	≤0.001	≤0.001


TNF-α (ng/mL)
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

in TNF-α production. Although all adrenergic treatments had significant effects, the overall increase in TNF-α production is not tremendous when compared to the media control.

Evaluating the above data for total TNF-α production by non-stimulated murine splenocytes over the two day time period using Tukey's pairwise comparison illustrates that there are significant differences between the media control treatment and some adrenergic treatments, but not others. The addition of anti-TNF-α antibodies to duplicate of conditioned media demonstrated that the effects illustrated throughout these experiments were due to TNF-α and thus proved that the WEHI-13VAR assay was able to measure the quantities of TNF-α secreted by the murine spleen cells.

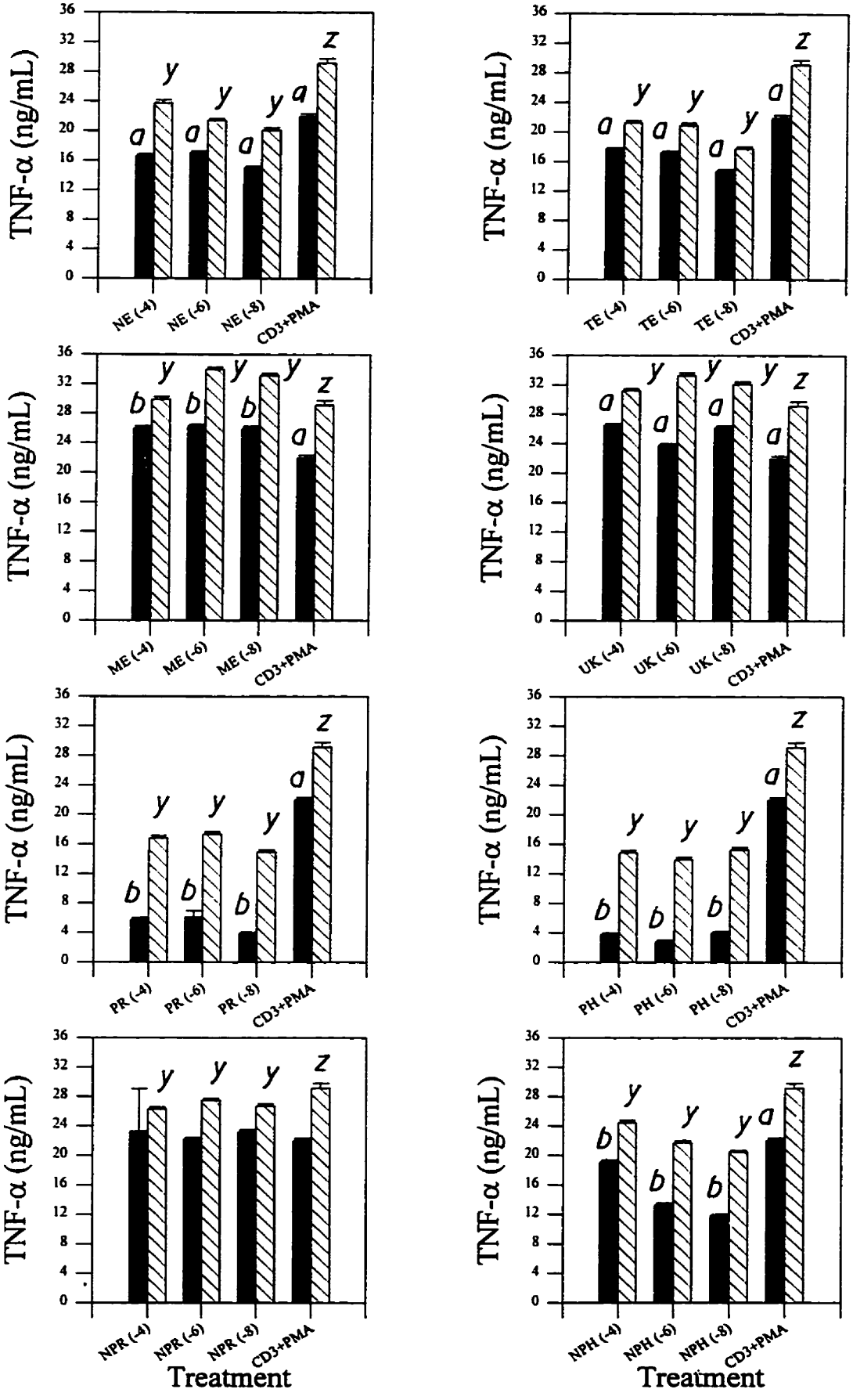
Adrenergic Agonist and Antagonist Treatments, CD3 + PMA Stimulated Murine Splenocytes and Tumor Necrosis Factor-α

The production of TNF-α by CD3 + PMA stimulated murine splenocytes in response to the adrenergic agonist and antagonist treatments was also examined over the concentration range of 10^{-4}, 10^{-6}, 10^{-8} M. Samples of the conditioned media were collected over a two day time course and assayed for TNF-α. The results of these experiments are displayed in Figure 7 -- 24 hr data is illustrated with solid bars, while the 48 hr data is represented by the hatched bars.

From preliminary observations, it is easy to again conclude that NE had significant effects on modulating the production of TNF-α by CD3 + PMA stimulated lymphocytes, as the production of total TNF-α was significantly inhibited when the splenocytes were co-cultured with NE and CD3 + PMA. Upon examination of the results utilizing the α-adrenergic agonists ME and UK, it is observed that both α-adrenoceptor treatments were individually able to significantly increase the production of TNF-α through their respective adrenoceptor targets. When the CD3 + PMA

FIGURE 7. A comparison of mean total TNF-α production over a two day time course by CD3 + PMA stimulated murine splenocytes treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the total TNF-α production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	PR	≤0.001	≤0.001
TE	≤0.001	≤0.001	PH	≤0.001	≤0.001
ME	≤0.001	≤0.001	NPR	≤0.001	≤0.001
UK	≤0.001	≤0.001	NPH	≤0.001	≤0.001


TNF-α (ng/mL)
NE (-4)
NE (-6)
NE (-8)
CD3+PMA
TE (-4)
TE (-6)
TE (-8)
CD3+PMA
ME (-4)
ME (-6)
ME (-8)
CD3+PMA
UK (-4)
UK (-6)
UK (-8)
CD3+PMA
PR (-4)
PR (-6)
PR (-8)
CD3+PMA
PH (-4)
PH (-6)
PH (-8)
CD3+PMA
NPR (-4)
NPR (-6)
NPR (-8)
CD3+PMA
NPH (-4)
NPH (-6)
NPH (-8)
CD3+PMA
Treatment
Treatment

stimulated splenocytes were treated with NPR, the β-adrenoceptor was blocked by PR and therefore the significant increases in TNF-α production observed, likely occurred through α-adrenoceptor modulation.

Subsequent experiments studying the effects of β-adrenoceptor modulation on TNF-α production by CD3 + PMA stimulated splenocytes illustrated that the β_2-adrenergic agonist terbutaline had significant inhibitory effects on the production of TNF-α. The effect of β-adrenoceptor inhibition on TNF-α production was also observed when NE was added in combination with PH (NPH). Thus from these results, it is apparent that NE can also target the β-adrenoceptor of the CD3 + PMA stimulated splenocyte to inhibit TNF-α production.

In summary, it is again apparent through these experiments that NE can modulate dose dependent effects on TNF-α production by CD3 + PMA stimulated murine splenocytes through the β-adrenoceptor, with the maximum total TNF-α production occurring at the maximum NE concentration. It is also apparent that NE can target the α-adrenoceptor to significantly increase TNF-α production by the stimulated murine splenocytes, however, these increases in TNF-α production do not necessarily reflect the dosage of the adrenergic treatment.

Evaluating the above data for total TNF-α production by CD3 + PMA stimulated murine splenocytes over the two day time period using Tukey's pairwise comparison illustrates that there are significant differences between the media control treatment and some adrenergic treatments, but not others. The addition of anti-TNF-α antibodies to duplicate samples of conditioned media again demonstrated that the effects illustrated throughout these experiments were due to TNF-α and thus proved that the WEHI-13VAR assay was able to measure the quantities of TNF-α secreted by the CD3 + PMA stimulated murine spleen cells.

DISCUSSION

The sympathetic nervous system is activated by both physiological and psychological stressors (99, 100, 101). Because much research of late has found innervation of many of the lymphoid organs and tissues by the sympathetic nervous system, research has begun determining the role of the nervous system in modulating immune function (98, 100, 101). This present research project was undertaken to determine the effects of norepinephrine on the production of TNF-α by two different cells lines, as well as by CD3 + PMA stimulated and non-stimulated murine splenocytes.

In previous murine splenic macrophage studies involving norepinephrine, it has been reported that norepinephrine is able to significantly increase as well as decrease TNF-α and TGF-β production. This effect has been found to depend both on adrenoceptor concentration and also on the concentration of NE. The significance of these findings are many, including the hypothesis that NE is able to affect the production of cytokines by lymphocytes through both α- and β-adrenoceptor modulation.

The β-adrenoceptor, Norepinephrine and Tumor Necrosis Factor-α Modulation

The results of this project illustrate that norepinephrine is able to significantly affect the production of TNF-α by the two transformed cell lines and murine splenocytes. Both of the transformed cell lines as well as the non-stimulated murine splenocytes produced significantly more TNF-α in the presence of NE while CD3 + PMA stimulated murine splenocytes produced significantly less TNF-α when co-cultured with NE.

Further studies utilizing the β_2-adrenergic agonist, terbutaline, illustrated very similar dose dependent effects to those observed with NE, notably, TE was able to significantly increase production of TNF-α to similar quantities in both populations of

transformed T cells. Even though the results utilizing the TE treatments were statistically conclusive, further studies are needed to establish if in fact the β_2-adrenoceptor subtype was responsible for mediating the observed response, since TE has affinity for both β_1 and β_2-adrenoceptors, but efficacy for the β_2-adrenoceptor (50).

The murine splenocytes which were treated only with an adrenergic agonist in the absence of CD3 + PMA stimulation displayed a significant increase in overall TNF-α production, however the production of TNF-α was not tremendously enhanced as compared to the control samples cultured only in media. The treatments of TE and NE again appear to have had similar concentration-dependent stimulatory effects on TNF-α production, thus it was concluded that the effect of norepinephrine modulation on TNF-α production by non-stimulated murine splenocytes was through the β-adrenoceptor and furthermore, the effect reflected the concentration of the agonist treatment.

In the CD3 + PMA stimulated murine splenocyte population, norepinephrine continued to have a modulatory effect on the production of TNF-α, although this effect was inhibitory rather than stimulatory. When both CD3 + PMA and the adrenergic agonist treatments were co-cultured with the murine splenocytes, NE and TE both similarly and significantly inhibited TNF-α production, and this inhibition accordingly appears to be dose dependent. Due to the similar nature of the observed effects of NE and TE, it was again concluded that NE was able to modulate a decrease in total TNF-α production through the β-adrenoceptor of the CD3 + PMA stimulated murine splenocyte.

The α-adrenoceptor, Norepinephrine and Tumor Necrosis Factor-α Modulation

When NE was added to the various cell samples, significant effects on TNF-α production were noted to occur through the β-adrenoceptor. However, because

norepinephrine can bind to either or both the α- and β- adrenergic receptors, it was necessary to determine what effect, if any, NE was modulating through the α-adrenoceptors. These experiments utilizing ME and UK (α_1- and α_2-adrenoceptor agonists, respectively) illustrate that production of TNF-α is significantly increased by all four cell samples through α-adrenoceptor modulation.

Both of the transformed cell lines had significant increases in TNF-α production when co-cultured with either of the α-adrenergic agonists and in fact, the quantities of TNF-α produced by the Jurkat and EL-4 cell lines stimulated with ME or UK are much greater than those obtained when the cell lines were treated with either NE or TE. From these results, it is apparent that not only can norepinephrine modulate TNF-α production through the α- or β-adrenoceptor, but also that the quantity of TNF-α produced depends upon the adrenoceptor target.

When examining the effects of the α-adrenoceptor agonist treatments on the production of TNF-α by non-stimulated and CD3 + PMA stimulated murine splenocytes, it is evident that UK and ME are individually able to significantly increase the production of TNF-α. In the non-stimulated population of murine splenocytes, TNF-α was again found to be significantly increased as compared to the media control population, however, TNF-α quantities were not tremendously enhanced above the baseline level.

The Adrenergic Antagonists, Norepinephrine and Tumor Necrosis Factor-α Modulation

Because of the adrenoceptor-dependent effects of NE, concurrent to the experiments with NE, TE, ME and UK, experiments studying the effect of NE in the presence of adrenergic antagonists were also performed. PH, an α-adrenergic antagonist, and PR, a β-antagonist were co-cultured with NE in a 1:1 ratio with the transformed and primary cell cultures. Since PR is a β-blocker it should eliminate any

effect of NE through the β-adrenoceptor, likewise since PH is an α-blocker, it should remove the modulatory effects of NE through the α-adrenoceptor.

In the populations of stimulated murine spleen cells, treatments of NPR had significant effects on the production of TNF-α as compared to the CD3 + PMA control treatment. This result suggests that NE is able to modulate a stimulatory effect on TNF-α production through the α-adrenoceptor, even when the β-adrenoceptor is not available. These results are confirmed by the increased TNF-α production observed when the same cell population was treated with the α-adrenergic agonists -- ME and UK. When the adrenergic treatment of NPH was co-cultured with the CD3 + PMA stimulated murine splenocytes, a significant decrease in TNF-α production was observed. Thus, it is apparent that NE modulates an inhibitory effect on TNF-α production when accessing only the β-adrenoceptor. This conclusion is again supported by the inhibition of TNF-α production which was observed when the TE was co-cultured with the CD3 + PMA stimulated murine splenocytes.

The collected data for the two transformed cell lines and non-stimulated murine splenocyte population also illustrates that NE when combined with either of the α- or β-adrenergic agonists was able to mediate similar effects to those observed with the adrenoceptor specific treatments of ME, UK and TE. In all three cultures, NE in combination with PH or PR produced a significant increase in TNF-α production, thus demonstrating that NE was able to modulate an effect even when the α- or β-adrenoceptors were respectively blocked.

When the antagonist treatments of PR and PH were individually added to the four cultures of cells, TNF-α production was significantly modulated. The addition of the treatments of PH and PR both produced a significant increases in TNF-α production by the EL-4 line as well as by the CD3 + PMA stimulated and non-stimulated murine splenocyte populations -- the PR treatments did not have conclusive results in modulating TNF-α production by the Jurkat cell line, however, the PH

treatments all significantly decreased the production of TNF-α in relation to cells treated with only media. These individual PR and PH treatments were intended only as drug controls, utilized to assure that the antagonists were not themselves having an effect on TNF-α production by the cell cultures. However, in the results, it is apparent that both antagonists were able to significantly modulate the production of total TNF-α. It is possible that the cells were releasing endogenous stores of NE, and when they were subsequently treated with an adrenergic blocker, the effect of the endogenous NE was modulated through the adrenoceptor which was not blocked by an adrenergic antagonist.

The Complex Role of Norepinephrine in Tumor Necrosis Factor-α Modulation by Transformed and Primary Cell Cultures

Very little research has focused to date on elucidating the role which NE plays in modulating the T cell response. In fact, this topic remains relatively unexplored in the literature, with little or no information being available to evaluate the results of this study. Therefore, only rudimentary hypotheses regarding the mechanisms through which the neurotransmitter norepinephrine functions to modulate the T cell immune response can be articulated.

Adrenergic receptors have typically been classified into four different types which show distinct patterns of selectivity to adrenergic agonists and antagonists (100, 103, 104, 105). At present, the four recognized receptors consist of the α_1, α_2, β_1 and β_2 subtypes (99, 100). Initially the presence of adrenergic receptors on cells was inferred from the effects of adrenergic agonists on cell function (99, 100). Gradually, as a variety of adrenergic agonists and antagonists became available, adrenergic receptors were additionally classified into groups according to the selectivity of the cellular response to the adrenergic agents as well as by adrenoceptor cloning experiments (99, 103, 104, 105).

It has been suggested in the available literature that T helper 1 and T helper 2 cells differentially express the β-adrenoceptor, and that the concentration of this receptor on these two T cell subsets does not change with activation (99, 100, 105). However, it has also been hypothesized that the β_2-adrenoceptor of activated Th1 and Th2 cells differentially modulates the production of cytokines as compared to non-activated Th1 and Th2 cells (100, 104, 105). Research has demonstrated that exposure of activated Th2 cell subsets to β_2-adrenoceptor agonists should induce no change in Th2 cell cytokine production, although exposure of activated Th1 cells to β_2-adrenoceptors decreases the production of some cytokines, including IL-2 (99, 100, 104). These previous experimental findings, therefore propose that differential expression of the β_2-adrenoceptor by activated Th1 and Th2 clones renders cytokine production by these clones differentially susceptible to the influence of the β_2-adrenoceptor ligand (99, 100, 104). Extremely little work has been presented regarding the potential existence of α-adrenoceptors on lymphocytes, although many studies have suggested that α-adrenergic agonists can modify T lymphocyte function (99, 104, 105). At present, the presence of α-adrenoceptors on T lymphocytes remains to be confirmed by ligand binding studies and thus remains a controversial topic in neuroimmunology (99, 100, 105).

Tumor necrosis factor-α is produced by T helper cells and is in fact produced by both the Th1 and Th2 subsets (89, 99, 105). The results obtained in this research project suggest that the murine splenocyte population which was stimulated with CD3 + PMA exhibited a β_2-adrenoceptor effect when NE was added, since the production of TNF-α was decreased. This is what is expected according to previously published research in a Th1 response since it is known, as previously stated, that activated Th1 cells exposed to a β_2-adrenoceptor ligand will decrease their production of cytokines (89, 99, 100). Therefore it appears that β_2-adrenoceptor agonists also have the same effect of inhibiting secretion of TNF-α by mixed murine splenocytes when co-

stimulated with NE and CD3 + PMA. Although an inhibitory response was present when utilizing a stimulated population of murine splenocytes, this response was contrary to that seen in the experiments performed with NE and non-stimulated splenocyte populations. Since there was no clear activation signal for any of the lymphocyte populations in the non-stimulated murine splenocyte sample, either a mixed response by the populations occurred to cause the increase in TNF-α production, or perhaps a population other than the T lymphocytes was undergoing an immune response to an unknown antigen.

The T cell lines, Jurkats and EL-4s, presented a very clear response to the addition of the adrenergic drug treatments. NE increased TNF-α production through the β-adrenoceptor, although the increase was not as great as was seen when the stimulus was through only the α-adrenoceptor. Although these β-adrenoceptor effects are contradictory to the previously published studies (89, 99, 100) relating to the decreased production of TNF-α by primary culture T cells stimulated with β_2-adrenoceptor agonists, it is feasible that there are reasons for the obvious discrepancies. Without characterization of the adrenoceptor types or concentrations on these two cell lines, it remains possible that both cell lines, due to their transformed lineage express an abundance of β-adrenoceptors and/or α-adrenoceptors and therefore their responses are not characteristic of primary culture tissues. Also, if a high concentration of β- and α-adrenoceptors are available, the modulatory effects of NE could be exacerbated, and furthermore, a mixed adrenoceptor response could be resulting in increased production of TNF-α. Without further study characterizing the effects of the α- and/or β-adrenoceptors, the deviations from previous research will remain, however the data accumulated in this research project overwhelmingly indicates that either an α- or β-adrenoceptor response results in increased TNF-α production by EL-4 and Jurkat cells, and this response occurs regardless of prior antigen stimulation.

Finally, this research project unexpectedly illustrated that PH and PR had significant modulatory effects on TNF-α production. Although this was not the anticipated effect of these control treatments, it remains plausible that these four cell types were able to secrete endogenous NE. At present, no research has been published regarding this phenomenon in lymphocyte populations, however, Spengler *et al.* (177) have studied this effect in macrophages. In this research by Spengler, it was noted that the β-adrenergic antagonist propranolol was able to augment the production of TNF-α, however, the α-adrenergic treatments of yohimbine and idazoxan had an inhibitory effect on TNF-α production (177). These results thus support the findings of extraneuronal accumulation of norepinephrine, however they do not determine the physiological reasoning for this system (177). Therefore, for the neuroimmunomodulation of TNF-α by lymphocytes to be studied completely, this hypothesis of endogenous norepinephrine production by these lymphocytes must be examined, as it may serve to support the role of neuroimmunomodulation in cytokine production as well as advance the possibility that norepinephrine can be released endogenously by the immune system to regulate immune responses.

Conclusions Regarding Tumor Necrosis Factor-α Modulation by Norepinephrine

In conclusion, the results of this project suggest that the sympathetic nervous system can act to inhibit T cell production of TNF-α by CD3 + PMA activated murine splenocytes through the β-adrenoceptor, and that this inhibition is most marked at submaximal concentrations of the catecholamine. It was also found that two transformed T cell lines produced increased amounts of TNF-α through both α- and β-adrenergic treatments, with the α-adrenergic treatment being a greater overall stimulator of TNF-α production. Although the latter result is contradictory to current published research regarding β-adrenoceptor inhibition of cytokine production, the

collected results from both the transformed and primary cultures indicate that the modulatory effects of norepinephrine depend not only on the presence of α- and/or β-adrenoceptors on lymphocytes, but that the resultant TNF-α production also reflects the concentration of the β-adrenergic drug.

CHAPTER III:

THE MODULATION OF TRANSFORMING GROWTH FACTOR-β BY NOREPINEPHRINE IN TRANSFORMED AND PRIMARY CELL CUTURES

Extensive research illustrating the interconnectedness of the nervous system and immune system through catecholamines has been demonstrated to play very important roles in many diseases like Idiopathic Pulmonary Fibrosis and cancer. This chapter focuses on determining the involvement of norepinephrine in the modulation of transforming growth factor-β (TGF-β) by four lymphocyte populations. T lymphocytes, from both transformed and primary cultures, were stimulated with adrenergic agonists and antagonists, including norepinephrine, and measurements were made to determine the effect and adrenoceptor target of norepinephrine in modulating TGF-β production. It was demonstrated that norepinephrine significantly augmented total TGF-β production by the non-stimulated murine splenocytes and both Jurkat and EL-4 cells lines in a dose dependent manner over a two day time course. This increased TGF-β production was modulated through both the α- and β-adrenoceptors of the lymphocytes. In the CD3 + PMA stimulated murine splenocyte population, the production of TGF-β was significantly inhibited by norepinephrine through β-adrenoceptor modulation, however, a significant increase in total TGF-β production was observed when α-adrenoceptor modulation occurred. When an analysis of the biological activity of the total TGF-β quantities was completed for all cell lines, it was determined that the CD3 + PMA stimulated murine splenocytes as well as the Jurkats and EL-4s produced mainly active TGF-β while the non-stimulated murine splenocytes produced predominately latent TGF-β. It is apparent through the use of various adrenergic agonist and antagonist treatments in this research project that the effect of norepinephrine on TGF-β production by T lymphocytes is dependent on both the adrenoceptor target and the dosage of the adrenergic treatment.

INTRODUCTION

An Introduction to Transforming Growth Factor-β

Often when cytokines are discovered, their original activity represents merely the tip of the iceberg (106, 107, 108). This was and continues to remain the case with

transforming growth factor-β. Not only is TGF-β able to participate in growth regulation, but it is also involved in many biological processes including, inflammation, host defense, tissue development and repair, immunoregulation and tumorigenesis (48, 106, 107, 109, 110, 111, 112). Transforming growth factor beta is an excellent example of a stable, multifunctional polypeptide growth factor involved in a multitude of immune reactions (108, 111, 113, 114, 115). The complex involvement of TGF-β in the immune response is relevant to normal development and repair processes of the body, as well as to various human disease conditions that are a direct result of excess or deficient levels of TGF-β (109, 111, 116).

Transforming growth factor-β can act either as an inhibitory or stimulatory molecule (107, 108, 111, 112). As a stimulatory molecule, TGF-β induces fibroblast proliferation, neutrophil chemotaxis as well as increased production of extracellular matrices (109, 110, 111). Its inhibitory effects include the inhibition of IL-1 receptor expression, suppression of the respiratory burst and a variety of cytotoxic effects directed towards activated macrophages (114, 118). TGF-β is also able to limit the immune function in inflammatory responses (112, 114, 117). The far ranging and diverse effects of TGF-β are thought to be a reflection of its many diverse physiological roles (111, 112, 117). Extensive literature has suggested that TGF-β plays a critical role in mediating a number of biological responses -- and that the response elicited depends not only on the binding of TGF-β, but also on the amount of TGF-β available, the state of development or differentiation of the cell, as well as other cytokines which may be present (48, 108, 112).

Transforming growth factor-β is one of many members of a large group of growth, differentiation and morphogenesis cytokines (118, 119, 120, 121, 122). This group of cytokines not only includes the TGF-β group, but also includes the activins and inhibins -- regulators of pituitary, gonadal and placental hormone production (118, 121, 122, 123). The members of this family of polypeptides share a common

homodimeric or heterodimeric structure and the biological similarity of this group within a vast span of vertebrate organisms underscores the high degree of evolutionary conservation of this cytokine (111, 124, 125). In fact, the conservation of amino acid sequences of TGF-β has been noted to be almost 100% between different mammalian species (42, 121, 124, 125).

Transforming growth factor-β is expressed and released by many cell types including: T cells, activated macrophages, platelets and B cells (42, 106, 122, 126). The TGF-β molecule consists of two 12 kDa disulfide linked polypeptide dimers and each highly conserved chain consists of approximately 112 amino acids (118, 124, 126, 127). At present, transforming growth factor-β has been found in five forms in mammals (118, 124, 126, 127). Of these five isoforms, TGF-β_1, TGF-β_2 and TGF-β_3 are highly homologous (125, 126, 128). Each TGF-β isoform contains an amino-terminal hydrophobic signal sequence which maintains the molecule in a latent state until the molecule is needed intracellularly, or serves to block the receptor-ligand interaction so that translocation into the lumen of the endoplasmic reticulum is halted (120, 126, 129). Preceded by a sequence of four basic amino acids, the bioactive domain of TGF-β, is located at the carboxy-terminus of the latent molecule (108, 120, 126, 129). Although pH and proteolysis can activate TGF-β *in vitro*, physiological activation of latent TGF-β by tumors is not completely defined, however a possible involvement of proteinases such as plasmin and cathepsin D has been suggested (108, 119, 120, 126). Once the 25 kDa molecule has been disassociated and is biologically active it can interact with TGF-β receptors identified on numerous cell types (120, 126, 130, 131).

Receptors for TGF-β have previously been identified as cell surface glycoproteins that exhibit high affinity and specificity toward the TGF-β molecule (132, 133, 134). Receptors are expressed ubiquitously, however in low concentrations by most normal and transformed mammalian cell types, and are able to discriminate

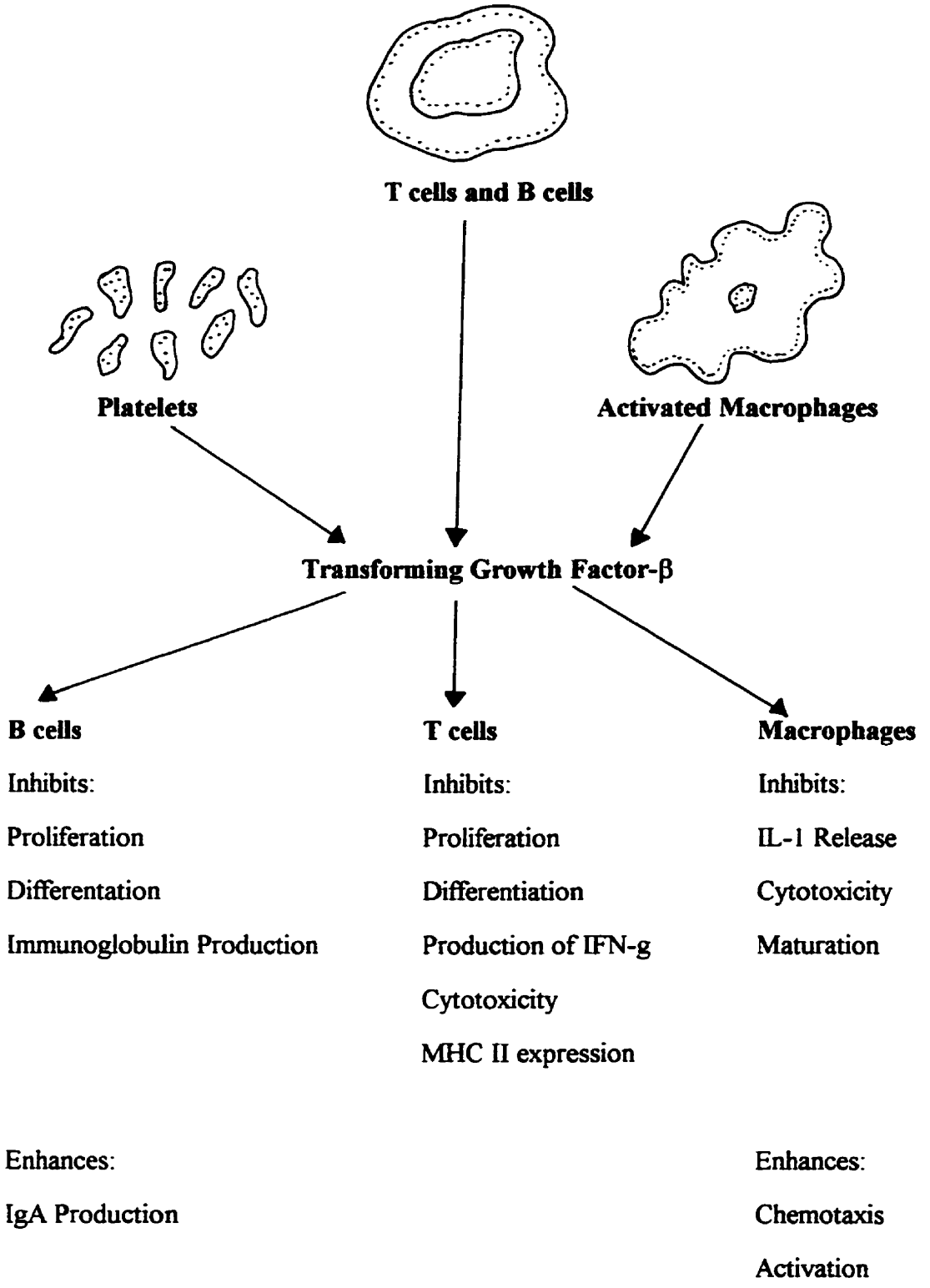


FIGURE 1. The origins and targets of Transforming Growth Factor-β.

between the various TGF-β isoforms (111, 134). Depending on cell type and function, there may be as few as 200 or as many as 100,000 TGF-β receptors per cell; analysis of the receptor binding data for a variety of cell types illustrates that cells with fewer receptors are able to bind TGF-β with a much higher affinity than cells with increased receptor concentrations (130, 132, 133, 134). These findings demonstrate that small amounts of TGF-β can have far reaching effects, suggesting that there are multiple cellular responses to TGF-β and thus accordingly there appear to be multiple pathways for signal transduction (132, 133, 134). While specific receptors have been found on almost all mammalian cells examined, the effect of TGF-β varies depending on cell type and growth conditions (111, 132, 134).

There is extensive literature suggesting that TGF-β plays a critical role in the mediation of a number of biological responses (48). One of the most characterized involvements of TGF-β in disease is within a group of fibrotic diseases recognized as the chronic pulmonary diseases (110, 118, 135). Chronic Pulmonary Fibrosis is characterized by fibrotic lung processes (118, 135) resulting from chronic infections and non-infectious inflammations (118, 135). Idiopathic Pulmonary Fibrosis (IPF) is one of the many chronic pulmonary fibrosis diseases, and is characterized by connective tissue deposition within the terminal air spaces of the lungs (118, 135, 136). The fatal result of this disease is due primarily to the overexpression of TGF-β by immune cells of these patients which is associated with increased collagen deposition (110). Thus, TGF-β plays an important if not primary role in the pathogenesis of IPF (110, 136).

TGF-β also appears to be a fundamental regulator of cell behaviour including tumour cells (43, 48, 137) with the nature of the response dependent on the cell type, growth conditions, the state of cell differentiation and the presence of other growth factors (111, 134). Elevated levels of this cytokine, as well as enhanced secretion of TGF-β have been observed in a number of human tumours, and it has been noted that

TGF-β is capable of promoting the invasion and metastasis of many cancers including, fibrosarcomas and mammary adrenocarcinomas (48, 137). Interestingly, TGF-β has a number of therapeutic benefits including the topical or systematic application of TGF-β for the healing of wounds, cartilage and bone repair, the suppression of inappropriate immune responses in autoimmune diseases, reversing or limiting transplant rejection as well as moderating the ischemic damage following heart attacks (123, 128, 138, 139).

The T Helper Cell Response and Transforming Growth Factor-β

With direct stimulation, most immune cells are able to secrete a variety of cytokines (26, 35, 86). These cytokines, acting as mediators, are able to both enhance interactions between immune cells and also serve as growth stimulators (26, 28, 86, 139). Transforming growth factor-β is produced by macrophages, T cells and B cells (26).

CD4+ effector Th cells are divided into two distinct subsets based on these cytokines -- Th1 cells are recognized as T cells which produce IL-2 and IFN-γ while Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13; both subsets of T cells can secrete a number of cytokines including IL-3, GM-CSF and TNF and TGF-β (28, 35, 86, 87, 88, 89). The cytokine production of these effector T helper cells essentially regulates the nature of most immune responses (28, 86, 87). Th1 cells are involved primarily in cell-mediated immune responses while Th2 cells function primarily in humoral immune responses (28, 86, 89). Although Th1 and Th2 cell responses are necessary for the maintenance of immune homeostasis, often the dominance of one response over another plays an important role in either the progression or clearance of many disease states (28, 86, 89).

Norepinephrine and Transforming Growth Factor-β -- A Relationship?

While previous evidence suggests a role for norepinephrine in immune regulation and modulation, little work has been performed studying this interaction as it relates to TGF-β (21, 89. 139). Because of the physical evidence of sympathetic innervation of lymphoid organs, and the close proximity these nerve terminals share with T cells, it seems plausible that norepinephrine could have an effect on T cell function (21, 89). It has been reported that norepinephrine, following immunization, stimulates the β_2-adrenoceptor on lymphocytes and that this stimulation in turn causes an increase in intracellular concentration of cAMP (21, 89). Thus it is possible that norepinephrine can modulate the β_2-adrenoceptor expression of T lymphocytes and may therefore also be able to modulate cytokine production.

At present, it has been hypothesized that norepinephrine does not modulate TGF-β receptor expression, however, an increase in adrenocorticotropic hormone or parathyroid hormone has been found to induce increases in TGF-β receptor binding (83). These findings also indicate that for some cells, TGF-β receptor expression is hormonally controlled (83). With the extensive literature suggesting that TGF-β plays a critical role in the mediation of a number of biological responses, it is obvious that its synthesis, secretion, activation and clearance must be tightly regulated and that defects in this regulation have potential for causing pathological conditions (83, 140). This chapter will focus on the role of norepinephrine in the modulation of TGF-β by T cells, both from transformed cultures and murine splenocyte samples.

An MTT Assay for Transforming Growth Factor-β

Because of the diverse spectrum of biological activities of TGF-β, a relatively wide choice of parameters for measuring TGF-β also exists (71, 96). Most commonly, TGF-β is quantified using a bioassay in which cell proliferation is either stimulated or inhibited (76, 91). These assays are effective, however, their reliability depends on the

TGF-β sensitive cell line chosen (76, 91). In addition, the assay must be stable over long periods of time and this is frequently a problem for the TGF-β assay as often cells lose their TGF-β sensitivity after continuous passage (76, 91).

Currently, although a dye-based TGF-β bioassay may be used, the most common method for quantifying this cytokine is through the uptake of radioactive [^{3}H]thymidine by the Mv1Lu cell line (71, 75, 114, 141, 142). However, the use of radioactive methods is expensive, time consuming and also proves difficult when processing a large number of samples (75, 138, 141). This research project utilized the MTT tetrazolium dye to quantify TGF-β inhibition of the A375 human malignant melanoma cell line (ATCC CRL1619). Using the procedures outlined for the [^{3}H]thymidine technique and Mv1Lu cell line as guidelines, the methods were modified, the cell line was changed, and the measurement method switched from a radioactive isotope to a tetrazolium dye (74). The result is an easy, quick, reliable method to measure TGF-β without concern for radioactive material usage (74).

The A375 assay for TGF-β is based on the cytotoxic effects of TGF-β on the A375 human malignant melanoma cell line. A375 viability is extremely limited in the presence of high concentrations of TGF-β and is relatively unaffected in the presence of low TGF-β concentrations. Utilizing MTT in combination with the A375 assay of TGF-β allows for the TGF-β content of the sample to be determined by comparing the conversion of the blue formazan product of the sample to a standard curve utilizing rTGF-β (74). Thus, the amount of formazan product is directly related to the cell viability after TGF-β sample addition, since only living cells can convert this yellow dye to its final product (74). Although this bioassay can be used to quantify TGF-β, it is unable to differentiate among the five isoforms. Therefore, in order to distinguish among isoforms, isoform-specific TGF-β antibodies must be added to duplicate samples to verify which isoform of TGF-β actually present.

MATERIALS AND METHODS

MATERIALS

A complete listing of the source and all types of media, solutions, specific chemicals and equipment used in this thesis, outlined in the methods section is attached as Appendix A.

METHODS

The methods utilized to perform the experiments studied in this chapter are as outlined in Chapter 2 with the exceptions noted below. The cytokine that was studied in this chapter was transforming growth factor-β.

Collection of Conditioned Media. After aliqouting the cells and the pharmacological reagents, the plates were allowed to incubate for a two day time course at 37°C, 95% CO_2, and at each of the two 24 hour intervals, collections of the conditioned media were made. Samples were collected in the presence of 1 μg/mL pepstatin, 0.5 μg/mL aprotinin and 0.5 μg/mL leupeptin in sterile, siliconized microfuge tubes and frozen at -80°C until ready for TGF-β quantification (94). These experiments were performed on each cell line and murine cultures a minimum of six times.

TGF-β assay. The TGF-β activity of the collected conditioned media was determined by the *in vitro* A375 assay based on a previously outlined procedure (71, 141, 142) with modifications and was expressed as total pg/mL for neutralized acidified conditioned media. A determination of % active versus % latent TGF-β was made through acidification of the conditioned media. Briefly, subconfluent A375 cells were used to assay the samples for TGF-β activity. Cells were washed, trypsinized and washed again with RPMI-1640 supplemented with 5% FBS. The collected cell

solution was centrifuged and the cell pellet resuspended in RPMI-1640 with 10% FBS, 1.0×10^{-3} M sodium pyruvate, 2.0×10^{-3} M L-glutamine, 5 mg/mL gentamycin sulfate and 4.5 mg/mL glucose and plated at a concentration of 1.5×10^3 cells/100 μL in a 96 well microtitre plate. Either neutral conditioned media, or conditioned media that was acidified and subsequently neutralized in the presence or absence of anti-TGF-β_1 antibodies was added at 100 μL/well, so that each of the wells contained a final total volume of 200 μL (119, 128, 143). Acidification of the neutral conditioned media was performed as described below.

After 90hrs of incubation at 37°C, 95% CO_2, 25 μL of a 5 mg/mL MTT solution was added to each well and the plate was allowed to further incubate at 37°C, 95% CO_2 for six hours (69, 70, 73, 74, 97, 144). The contents of the wells were then carefully removed by gently inverting and blotting the microtitre plate (70, 73, 74). Subsequently, after 100 μL/well of a stop solution of acidified SDS was added to each well, the plate was vigorously shaken for 25 minutes, and finally the absorbance was read in a 750 Cambridge plate reader at 590 nm (70, 72, 73, 74). The activity of the TGF-β in the samples was calculated using an rTGF-β standard (R&D) prepared at the same time as the experimental samples.

Prior to the use of the A375 assay for the measurement of TGF-β, the assay was calibrated (71). The cell number which would give maximal OD readings at 590 nm was selected, and a standard of rTGF-β was defined to be utilized each time the assay was run; this pre-defined standard from 5000 pg/mL rTGF-β to 0 pg/mL TGF-β was used to verify the sensitivity of the assay throughout the experiments (71).

Also prior to the use of the A375 bioassay for quantifying TGF-β, the adrenergic drug treatments were added to the A375 cell line to determine whether or not the adrenergic drug treatments had an effect on the A375 cells. After plating the A375 cells, 100 μL of each adrenergic drug treatment at the maximum experimental concentration (10^{-4} M) was added. The plates were allowed to incubate, read, and a

comparison of the optical density readings were made to A375 cells which received no adrenergic drug treatment. It is imperative that the A375 cells be sensitive only to the TGF-β content of the sample and not to the adrenergic drug treatment.

When the assay was utilized to quantify TGF-β in collected supernatant samples, the samples were dividied so that triplicate repeats could be performed. After obtaining all data, the mean of the data was determined as well as a calculation of the standard deviation of the mean.

Acidification and Neutralization of TGF-β conditioned media samples. Both neutralized and acidified/neutralized samples must be run to quantify TGF-β levels since TGF-β has two isoforms -- a biologically active and a biologically latent form. Samples were divided and placed in siliconized microfuge tubes. To the sample which was to be acidified and therefore quantify total TGF-β (both latent and active TGF-β), 10 μL of a 1.8 N HCl was added per 400 μL of sample (141). The sample was mixed well and allowed to stand for 5 minutes. Promptly at five minutes, 16 μL of solution D per 400 μL sample (Appendix A) was added, the sample was again mixed well and subsequently added to the A375 cells (141). The sample which was to quantify active TGF-β was added without acidification to the A375 cells. After the quantification of TGF-β using the A375 cells, the active TGF-β quantity was subtracted from the total TGF-β levels to give the amount of latent TGF-β present (141).

TGF-β Statistical analysis. The data are expressed as total TGF-β content as well as % active and % latent of total TGF-β content. Values were compared using the Minitab Statistical Software for ANOVA. All values are reported as the mean ± the standard deviation and analyzed by analyzed by Tukey's pairwise comparison test (n=6). An alpha error of 0.05 was used to test for statistical significance.

RESULTS

The Transforming Growth Factor-β MTT Bioassay and Calibration

Effects of cell number on formazan production by A375 cells, a human malignant melanoma cell line in combination with a rTGF-β standard. To determine the effect of cell number on MTT conversion to blue formazan, five concentrations of A375 cells were selected for pre-testing with a shortened rTGF-β standard. The cell number ranged from the highest concentration of 1.0×10^6 cells/mL to the lowest concentration of 1.0×10^4 cells/mL. A short standard of rTGF-β was prepared ranging from 0 pg/mL to 5000 pg/mL and was added to the cells to incubate for 96 hours. Following a further incubation with MTT dye, the plates were read and a comparison of the optical density readings was made. A wide range between the optical density readings of the maximal rTGF-β concentration and the 0 pg/mL rTGF-β is important so as to provide a high level of sensitivity for the assay; this wide range was present when the cell concentration of 1.5×10^4 cells/mL was utilized, therefore this cell concentration was chosen for subsequent TGF-β assay procedures (Figure 2).

Effects of adrenergic agonist and antagonist treatments on the A375 cell line. To determine the effect of the adrenergic agonist and antagonist treatments on the A375 cell line, all adrenergic agonists and antagonists were co-incubated at their maximum concentration (10^{-4} M) with the A375 cell line at the same point where sample addition would occur in the TGF-β assay procedure. The results from this experiment are illustrated in Figure 3. The results demonstrate that the adrenergic treatments did not have an effect on the A375 cell line as compared to A375 cells treated in the absence of an adrenergic treatment, as all optical density readings are similar.

FIGURE 2. A TGF-β standard curve utilizing A375 cells at a concentration of 1.5 x 10^4 cells/mL. A short standard from 0 ng/mL to 2500 pg/mL rTGF-β was prepared and sampled with five concentrations of A375 cells. The varying concentrations of A375 cells displayed different optical density readings over the same rTGF-β standard. The samples containing the A375 at a concentration of 1.5 x 10^5 cells/mL gave the greatest difference in optical density readings over the 0 ng/mL to the 50 ng/mL rTGF-β concentrations. The rTGF-β standard curve obtained for this cell concentration utilizing the defined standard is displayed. Each point on the graph represents the mean of a triplicate and is shown with error bars noting the standard deviation of the mean.

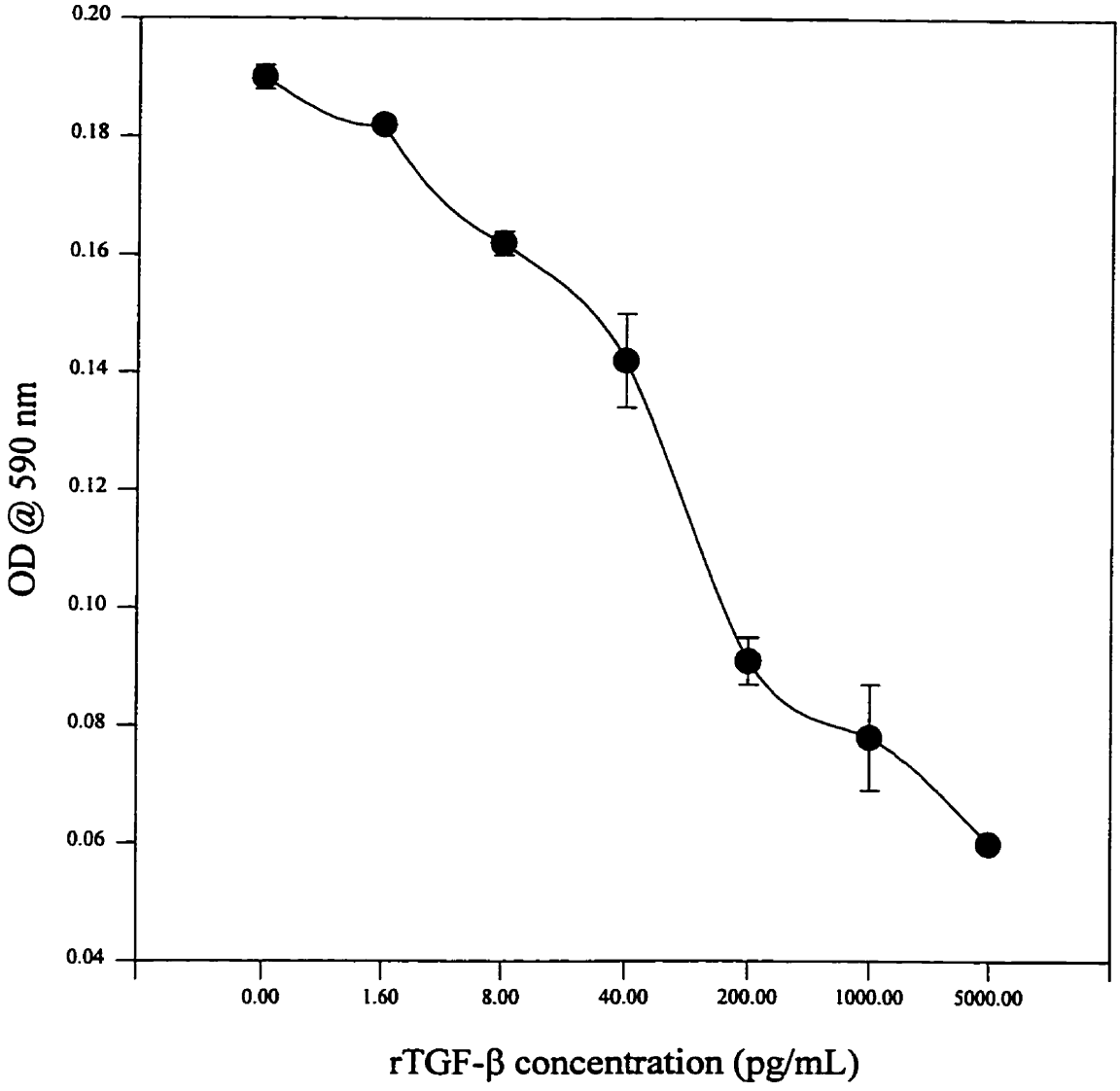
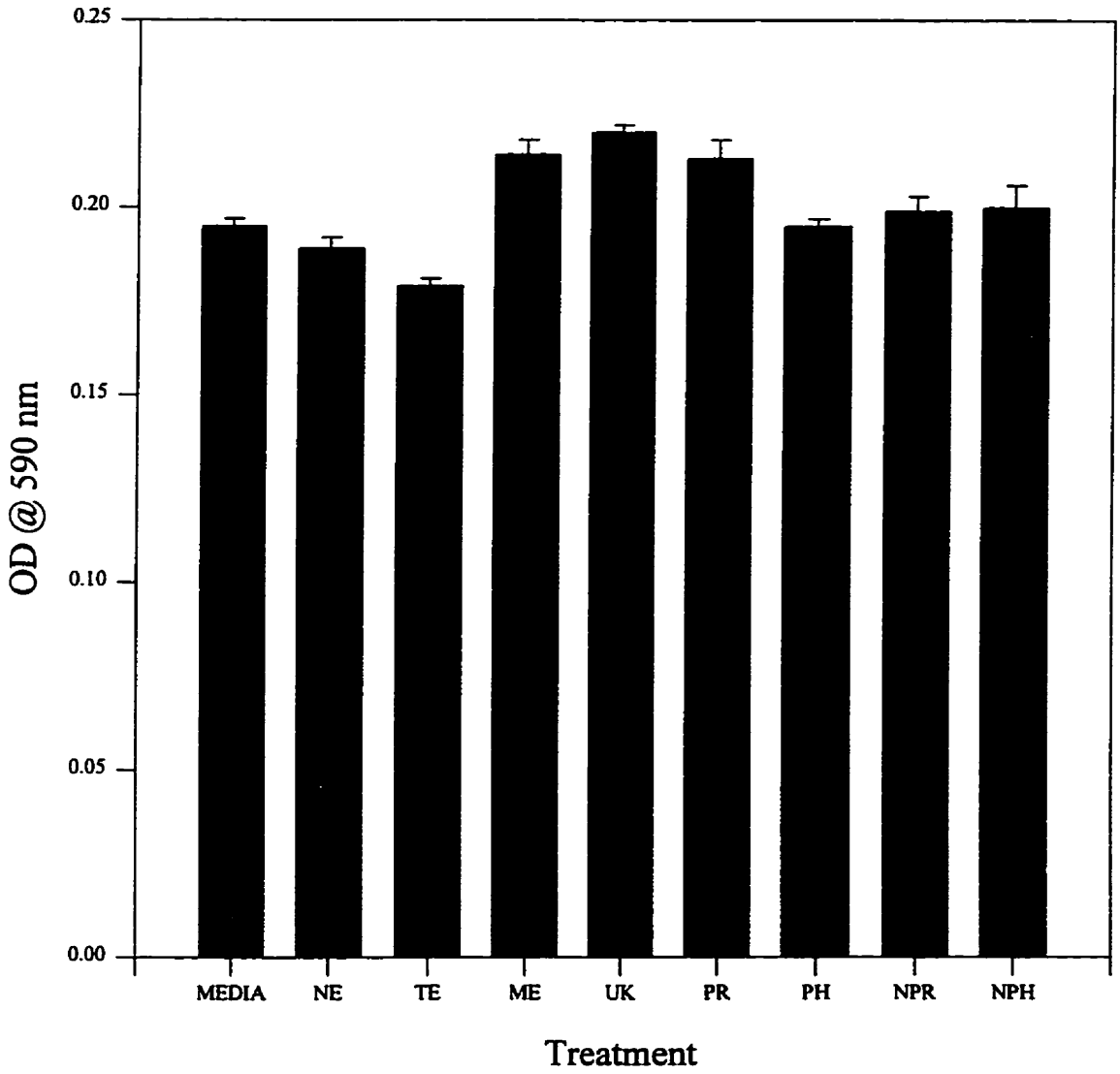

0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
OD @ 590 nm
0.00
1.60
8.00
40.00
200.00
1000.00
5000.00
rTGF-β concentration (pg/mL)

FIGURE 3. Effects of adrenergic agonist and antagonist treatments on the A375 cell line. The adrenergic agonist and antagonist treatments were added to the A375 cell line after the first overnight incubation to test the effects of the various adrenergic treatments on the A375 cells. This addition of adrenergic treatments took place at the same time point as the addition of conditioned media samples would occur in the TGF-β assay procedure. The different adrenergic treatments did not have any significant effect on the optical density readings as compared to A375 cells treated only with media ($p \leq 0.001$). Each point on the graph represents the mean of a triplicate and is shown with error bars noting the standard deviation of the mean.


0.25
0.20
0.15
0.10
0.05
0.00
OD @ 590 nm
MEDIA
NE
TE
ME
UK
PR
PH
NPR
NPH
Treatment

Adrenergic Agonist and Antagonist Treatments, Jurkat Cells and Transforming Growth Factor-β

The production of TGF-β by Jurkat cells in response to adrenergic agonists and antagonists was measured over a two day time course over a concentration range of 10^{-4}, 10^{-6}, 10^{-8} M. Samples of conditioned media were collected over a two day time course and assayed for TGF-β. The results of these experiments are displayed in Figure 4 -- 24 hr data is illustrated with solid bars, while the 48 hr data is represented by the hatched bars.

In the experimental results, it is noted that NE can increase significantly the production of total TGF-β by Jurkat cells. Since NE can act through both α- and β-adrenoceptor types, and because cells of the immune system are known to have both types of these receptors, the effects of the individual adrenoceptors in response to NE was examined with the adrenoceptor specific agonists and antagonists; TE, ME, UK, PR and PH. When the β_2-adrenoceptor of the Jurkats was directly stimulated with TE, total TGF-β production by the Jurkat cells was significantly increased. Furthermore, when the ability of NE to target the α-adrenoceptor was eliminated with PH (NPH treatment), total TGF-β production was again significantly augmented. The results of the TE and NPH treatments, when combined, suggest that NE can significantly increase total TGF-β production through the β-adrenoceptor of the Jurkat cell line.

Subsequently, an investigation of the α-adrenoceptor effects of NE was performed utilizing ME and UK, α_1- and α_2- adrenergic agonists, respectively. By directly stimulating the α_1-adrenoceptor with ME, a significant increase in total TGF-β production was observed. Similarly, the α_2-adrenoceptor when stimulated with UK, also produced a significant increase in total TGF-β production. When utilizing the NPR treatments, NE modulated a significant increase in total TGF-β production, and this effect should have been through the α-adrenoceptor, as the β-adrenoceptor was

FIGURE 4. A comparison of mean total TGF-β production over a two day time course by Jurkat cells treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the total TGF-β production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	PR	≤0.001	≤0.001
TE	≤0.001	≤0.001	PH	≤0.001	≤0.005
ME	≤0.001	≤0.001	NPR	≤0.001	≤0.001
UK	≤0.001	≤0.001	NPH	≤0.001	≤0.001

Adrenergic Treatment Abbreviations:

NE:	Norepinephrine	PR:	Propranolol
TE:	Terbutaline	PH:	Phentolamine
ME:	Methoxamine	NPR:	Norepinephrine + Propranolol
UK:	UK14304	NPH:	Norepinephrine + Phentolamine

Adrenergic Treatment Concentrations:

(-4):	10(-4) M	(-6):	10(-6) M	(-8):	10(-8) M

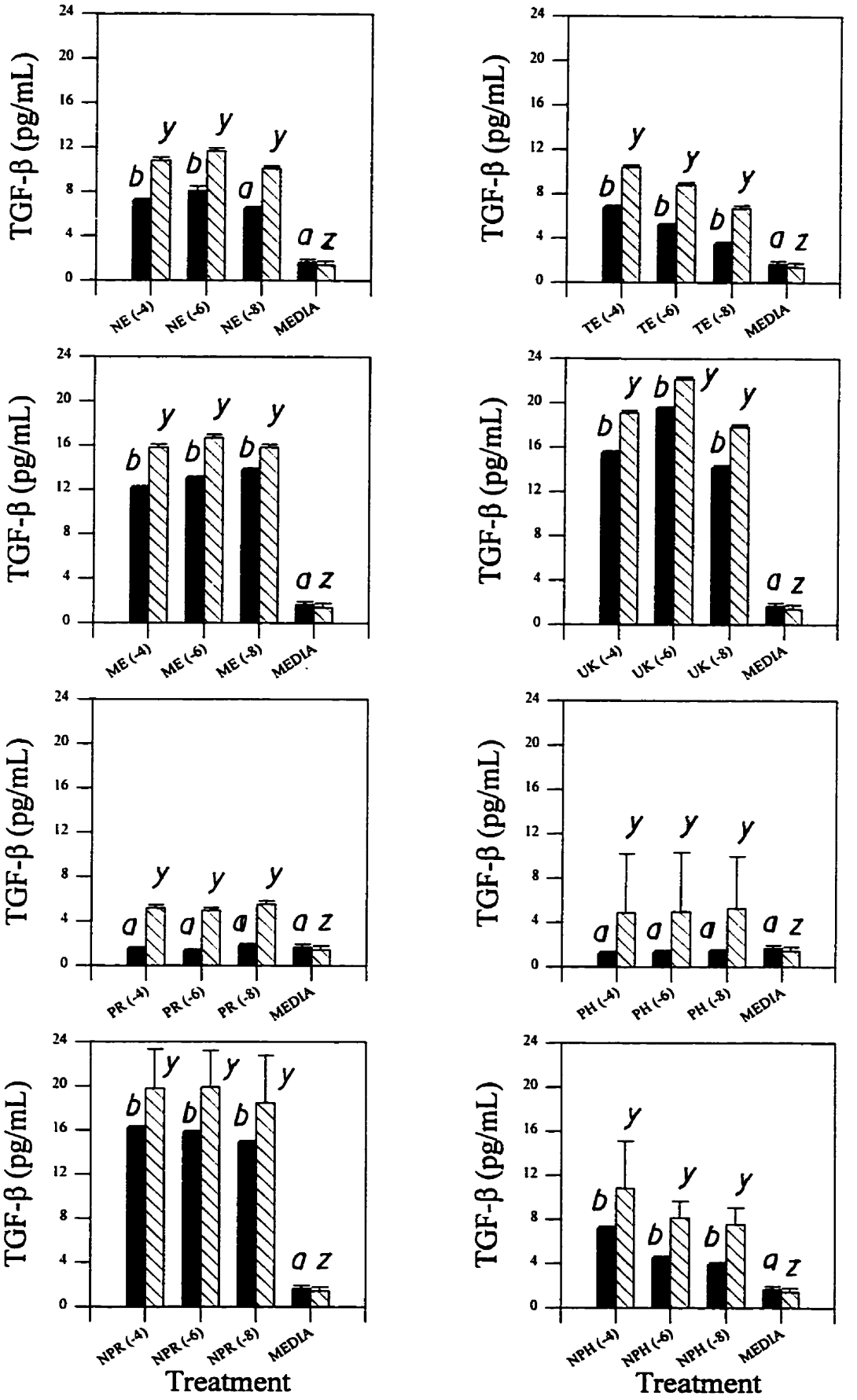
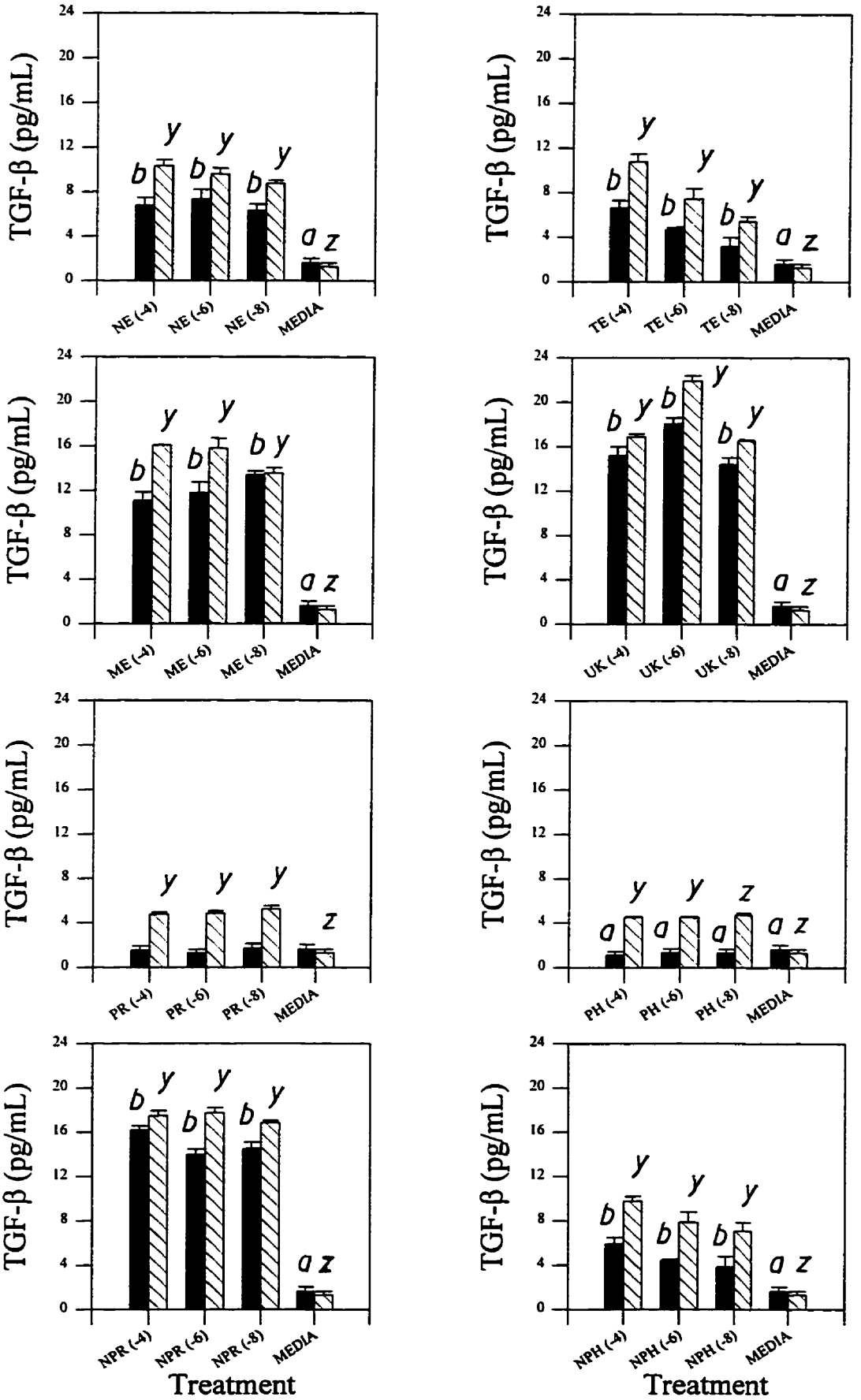

TGF-β (pg/mL)
24
20
16
12
8
4
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

FIGURE 5. A comparison of mean active TGF-β production over a two day time course by Jurkat cells treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the active TGF-β production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	PR	≤0.304	≤0.001
TE	≤0.001	≤0.001	PH	≤0.111	≤0.001
ME	≤0.001	≤0.001	NPR	≤0.001	≤0.001
UK	≤0.001	≤0.001	NPH	≤0.001	≤0.001


TGF-β (pg/mL)
24
20
16
12
8
4
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

blocked by the PR. Examining the results of the UK, ME and NPR treatments as a group, suggests that NE can significantly increase total TGF-β production through the α-adrenoceptor of the Jurkat cell.

The effects of each of the adrenergic antagonists on total TGF-β production were also determined through the use of individual PR and PH treatments. These results illustrate that both PH and PR were individually able to increase significantly the production of total TGF-β by Jurkat cells at 24 and 48 hrs.

In summary, these results suggest that NE can target both the α- and β-adrenoceptor to increase significantly the production of total TGF-β. The results illustrate that NE may have some preference for the β-adrenoceptor, as the effects of NE on total TGF-β production were similar to those observed with the TE treatments. NE, when targeting the β-adrenoceptor also appears to modulate a dose dependent effect with higher concentrations of NE producing greater quantities of total TGF-β. The results also illustrate that NE can act through either the α_1- and α_2-adrenoceptor subsets to modulate increased total TGF-β production, however, this effect does not appear to relate solely to the concentration of the adrenergic treatment.

Through an examination of the effects produced by the various adrenergic treatments on active TGF-β production by the Jurkat cells (Figure 5), it was observed that active TGF-β production by the Jurkat cell line was significantly increased over the two time course points by most adrenergic treatments.

Evaluating the above data for total TNF-α production by Jurkat cells over the two day time period using Tukey's pairwise comparison illustrates that there are significant differences between the media control treatment and some adrenergic treatments, but not others (Figure 4 and Figure 5, respectively). The addition of anti-TGF-β_1 antibodies to duplicate samples of conditioned media demonstrated that the effects illustrated throughout the experiments were due to TGF-β_1 and thus the A375

assay was able to measure the quantities of TGF-β secreted by the Jurkat cells (119, 128, 133, 151, 170).

Effects of adrenergic agonist and antagonist treatments on the production of biologically latent and active TGF-β by Jurkat cells. TGF-β was quantified both before and after activation by acidification because TGF-β obtained from most cells, including platelets and tissues is not normally in a biologically active form (141). The results of the acidification process for the total TGF-β secreted by the Jurkat cells (Figure 4) are displayed as means in Figure 6A and B for the 24 hr data and the 48 hr data, as % latent TGF-β (hatched bars) versus % active TGF-β (black bars) respectively. It is visible through the results that predominantly all TGF-β secreted by Jurkat cells was in an active form.

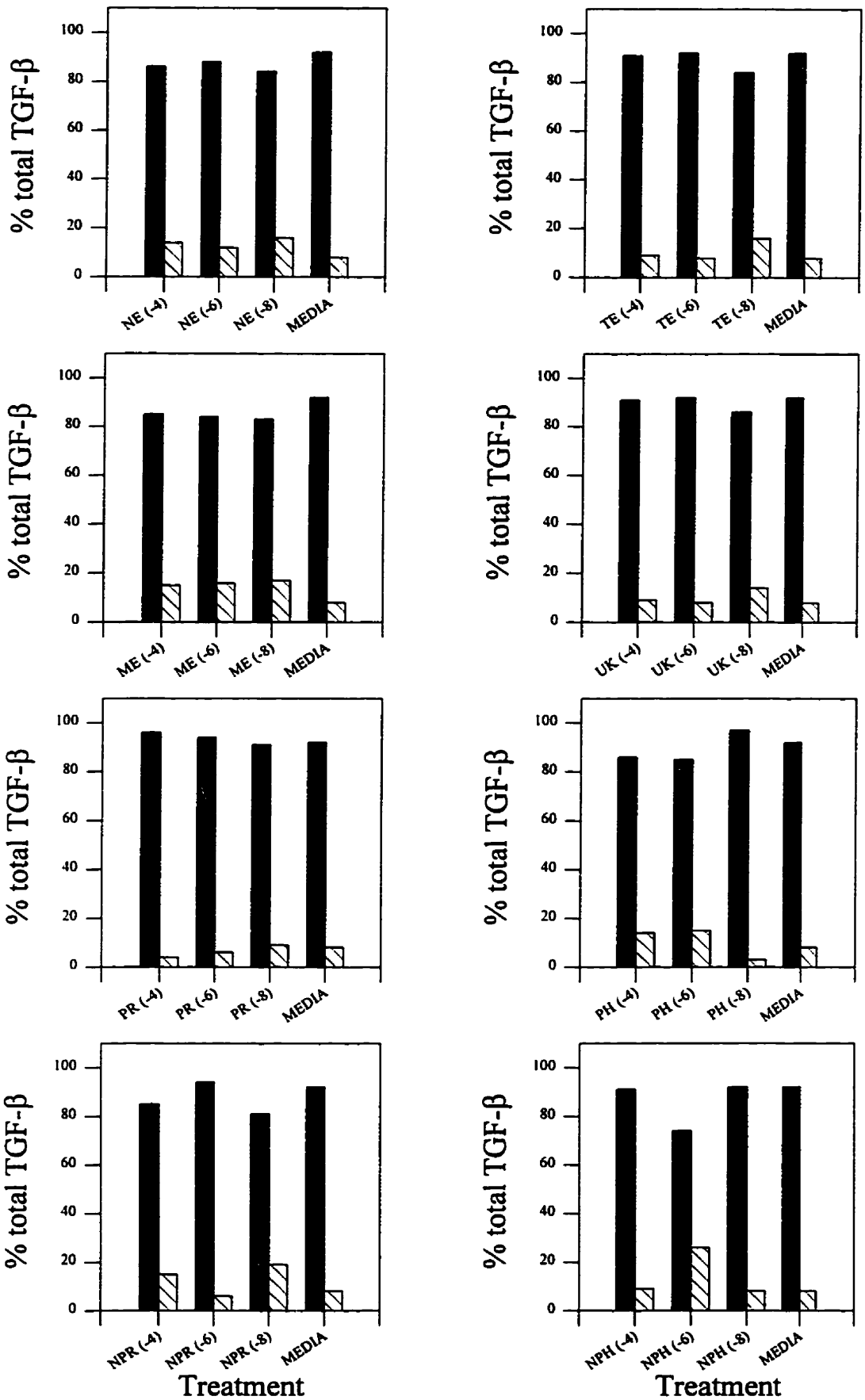
Adrenergic Agonist and Antagonist Treatments, EL-4 Cells and Transforming Growth Factor-β

The production of TGF-β by EL-4 cells in response to NE, TE, ME, UK, PR, PH, NPR and NPH was also tested over the concentration range of 10^{-4}, 10^{-6}, 10^{-8} M. Samples of conditioned media were collected over a two day time course and assayed for TGF-β. The results of these experiments are displayed in Figure 7 for total TGF-β and Figure 8 for active TGF-β. In each of these figures, the 24 hr data is illustrated with solid bars, while the 48 hr data is represented by the hatched bars.

From these preliminary results, it can be observed that NE can significantly increase the production of total TGF-β by EL-4 cells. Again, because of NE's dual ability to act through both α- and β-adrenoceptors, the effects of the individual adrenoceptors in response to NE were measured. Targeting the β_2-adrenoceptor of the EL-4s with the TE treatments produced a significant increase in total TGF-β production. When the α-adrenoceptor was subsequently blocked with PH in the NPH

FIGURE 6. The biological activity of TGF-β produced by Jurkat cells treated with various adrenergic agonists and antagoinsts. Based on the total TGF-β concentrations obtained through experimentation (Figure 3), calculations of % latent TGF-β (hatched bars) compared to % active TGF-β (shaded bars) were completed. Figure 5A demonstrates the mean activity of the TGF-β in the samples collected at the 24 hour time point and Figure 5B -- the mean activity at 48 hrs.

A


% total TGF-β
100
80
60
40
20
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

B

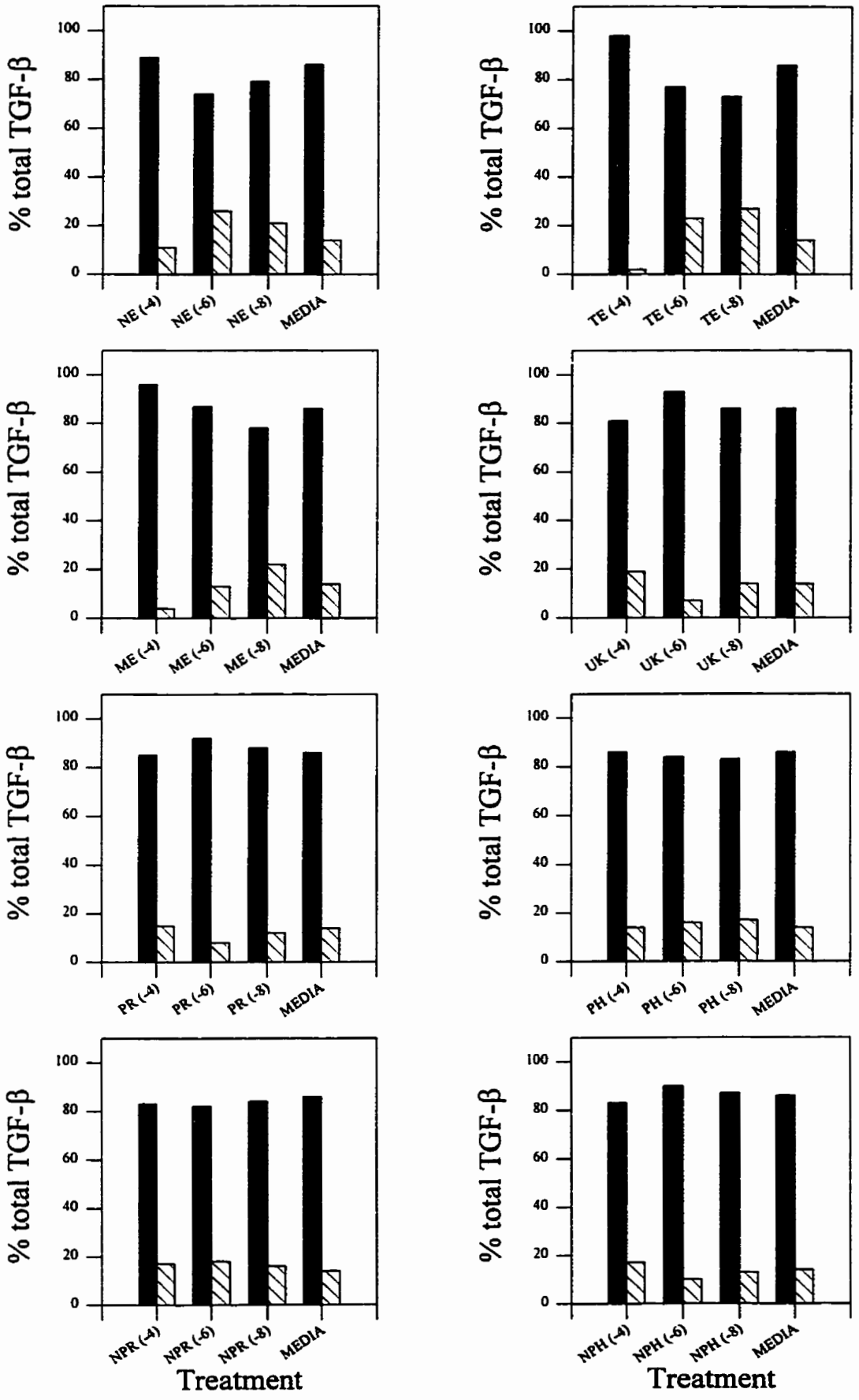
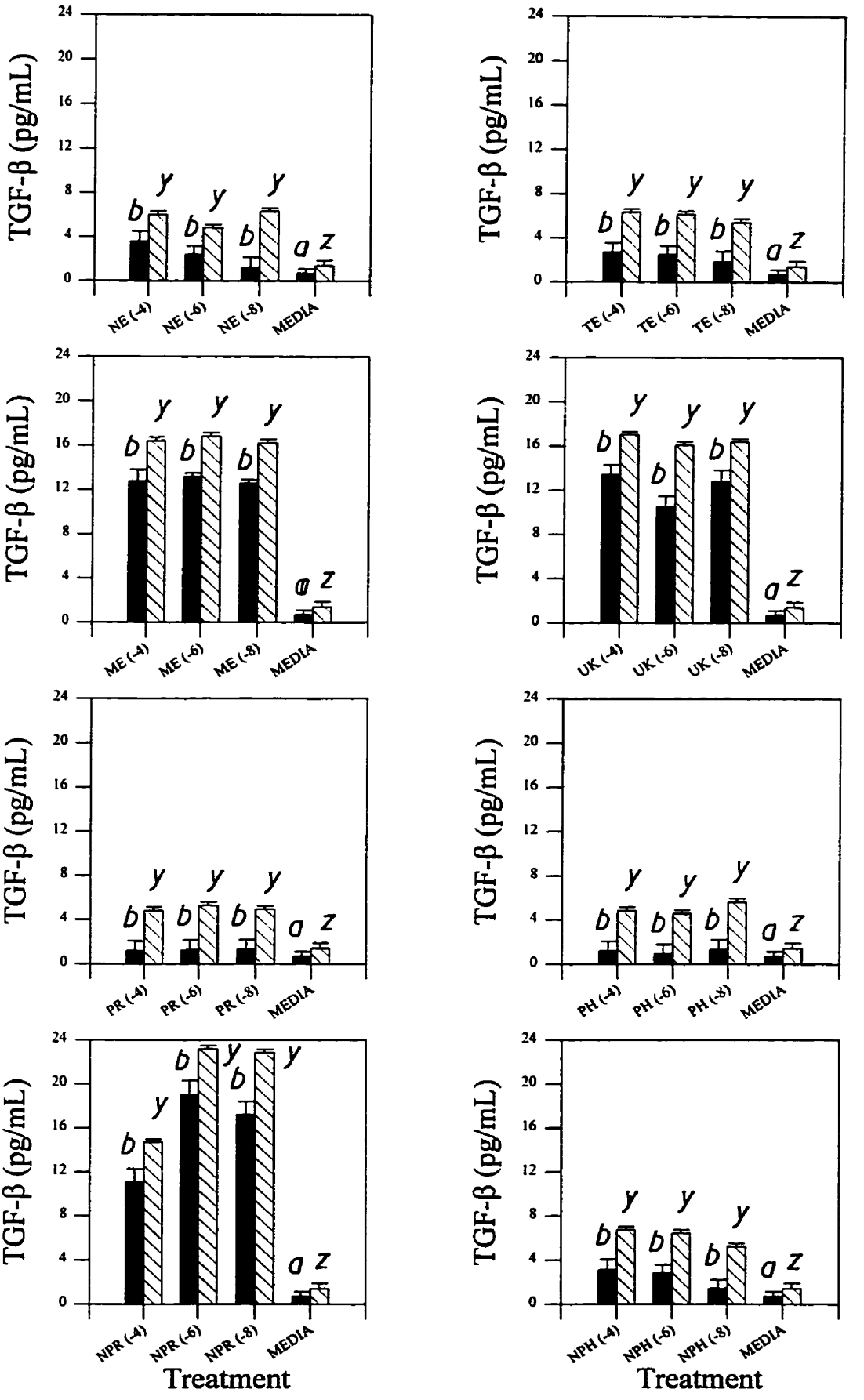

% total TGF-β
100
80
60
40
20
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

FIGURE 7. A comparison of mean total TGF-β production over a two day time course by EL-4 cells treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the total TGF-β production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	PR	≤0.001	≤0.001
TE	≤0.001	≤0.001	PH	≤0.001	≤0.001
ME	≤0.001	≤0.001	NPR	≤0.001	≤0.001
UK	≤0.001	≤0.001	NPH	≤0.001	≤0.001


TGF-β (pg/mL)
24
20
16
12
8
4
0
b
y
a
z
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

treatment, the production of total TGF-β was again observed to be significantly increased. This combined set of results proposes that NE can significantly increase total TGF-β production when accessing the EL-4 cell through the β-adrenoceptor.

Further studies with the ME and UK treatments examined the specific α–adrenoceptor effects of NE on modulating total TGF-β production. Stimulating the α_1-adrenoceptor with ME resulted in a significant increase in total TGF-β production. Likewise, when UK was utilized to stimulate the α_2-adrenoceptor, total TGF-β production was again significantly increased. When the ability of NE to target the β-adrenoceptor of the EL-4 cell was eliminated through the use of PR (the NPR treatment), NE was able to significantly increase the total TGF-β production. These results, examining the effects of α-adrenoceptor modulation by UK, ME and NPR, suggest that NE is able to increase total TGF-β production significantly through the α-adrenoceptor of the EL-4 cell.

Finally, the individual effects of each of the adrenergic antagonists on the production of TGF-β by EL-4 cells was studied . From these results, it is noted that PR and PH treatments were individually able to significantly increase the production of total TGF-β by the EL-4 cell line at 48 hrs, but not at the 24 hr time course point.

In conclusion, the data suggests that NE can target either the α- and β-adrenoceptor of the EL-4 cell line to modulate an increase in total TGF-β production. It again appears that NE may have preference for the β-adrenoceptor since the effects of NE on EL-4 total TGF-β production were similar to those observed with the TE treatments; this effect also appears to be dose dependent with increased TGF-β quantities produced at the higher adrenergic drug concentrations. It was also noted that NE can modulate increased total TGF-β production through the α-adrenoceptor, however, these effects do not necessarily reflect the concentration of the adrenergic drug.

The adrenergic agonist and antagonist treatments appear to have had some significant effects on active TGF-β production by the EL-4 cells (Figure 7). It is again observed that active TGF-β production was significantly increased at the 24 and 48 hour time periods by most adrenergic treatments.

Evaluating the above data for total TNF-α production by EL-4 cells over the two day time period using Tukey's pairwise comparison illustrates that there are significant differences between the media control treatment and some adrenergic treatments, but not others (Figure 7 and Figure 8, respectively). The addition of anti-TGF-β_1 antibodies to duplicate samples of conditioned media demonstrated that the effects illustrated throughout the experiments were due to TGF-β_1 and thus the A375 assay was able to measure the quantities of TGF-β secreted by the EL-4 cells.

Effects of adrenergic agonist and antagonist treatments on the production of biologically latent and active TGF-β by EL-4 cells. TGF-β was again quantified both before and after activation by acidification. The results of the acidification process of the total TGF-β obtained in Figure 6 are observed in Figure 9 -- A and B for the 24 and 48 hr time course respectively. The results are displayed as % latent TGF-β (shaded bars) versus % active TGF-β (black bars). It is again visible through the results that predominantly all TGF-β secreted by the EL-4 cells was in an active form.

Adrenergic Agonist and Antagonist Treatments, Non-Stimulated Murine Splenocytes and Transforming Growth Factor-β

The production of TGF-β by non-stimulated murine splenocytes in response to the adrenergic agonist and antagonist treatments was also tested over the concentration range of 10^{-4}, 10^{-6}, 10^{-8} M. Samples of the conditioned media were collected over a two day time course and assayed for TGF-β. The results of these experiments are displayed in Figure 10 for total TGF-β and Figure 11 for active TGF-

FIGURE 8. A comparison of mean active TGF-β production over a two day time course by EL-4 cells treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the active TGF-β production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	PR	≤0.001	≤0.001
TE	≤0.001	≤0.001	PH	≤0.007	≤0.001
ME	≤0.001	≤0.001	NPR	≤0.001	≤0.001
UK	≤0.001	≤0.001	NPH	≤0.001	≤0.001

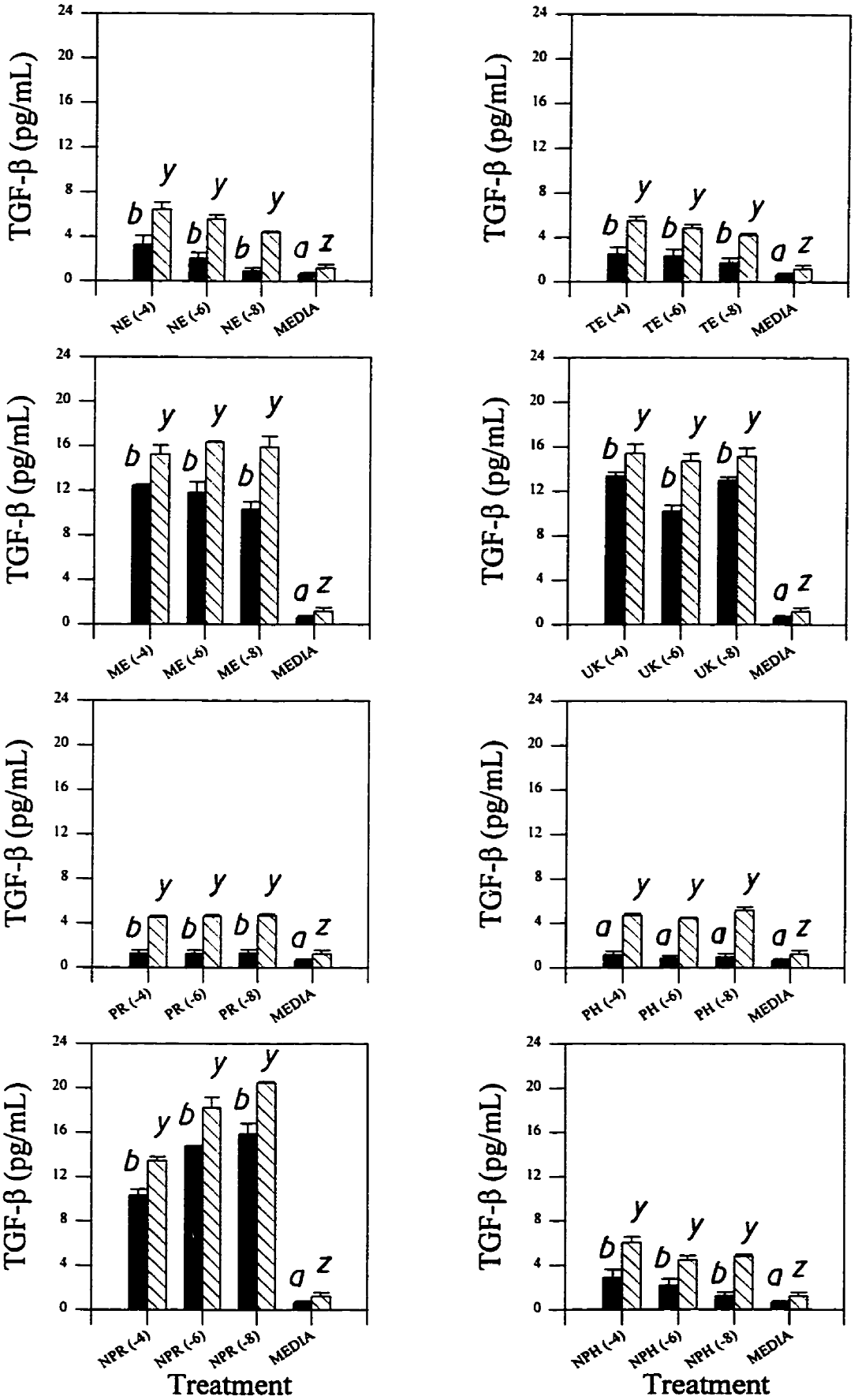
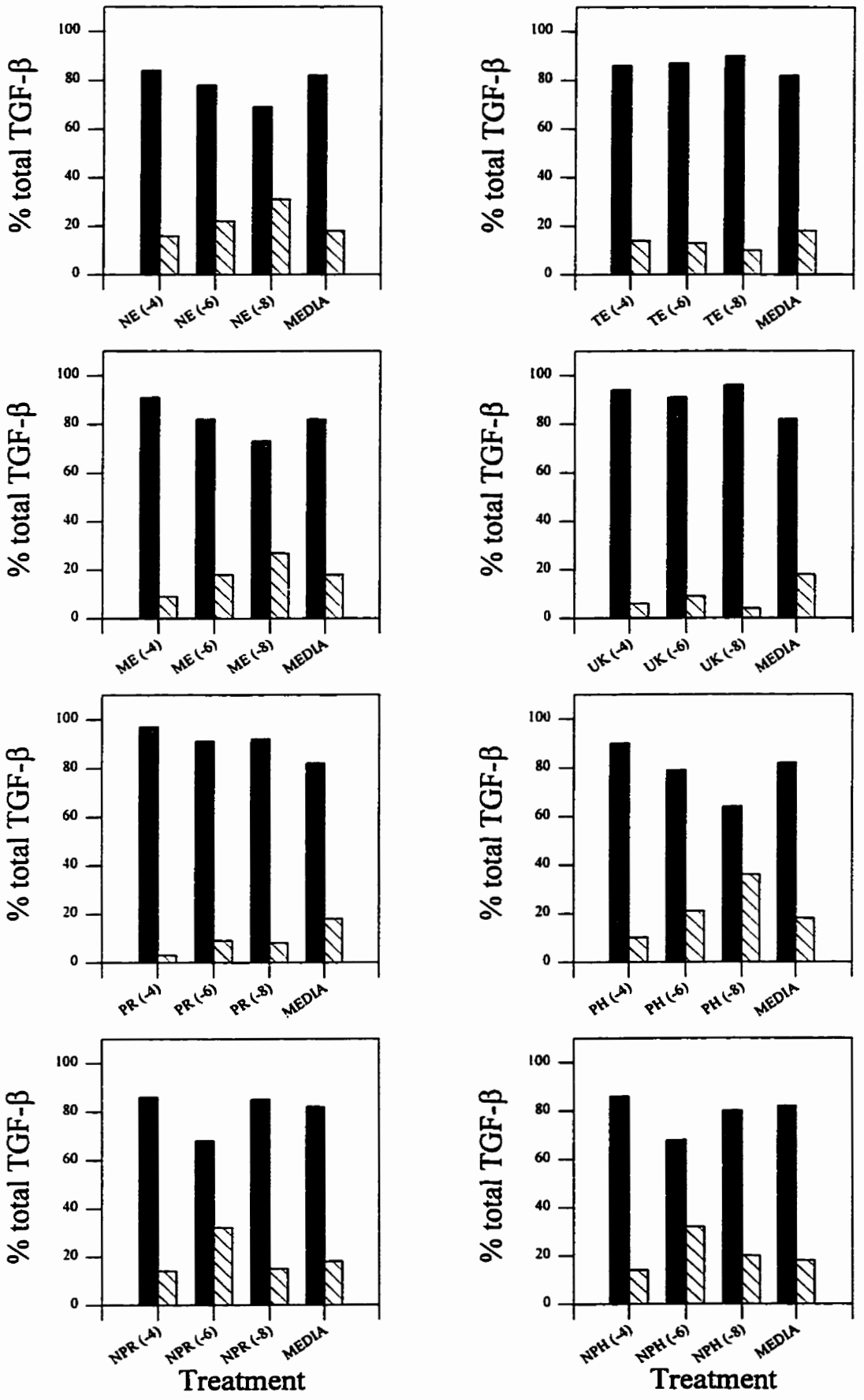

TGF-β (pg/mL)
24
20
16
12
8
4
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

FIGURE 9. The biological activity of the TGF-β produced by EL-4 cells treated with various adrenergic agonists and antagoinsts. Based on the total TGF-β concentrations obtained through experimentation (Figure 6), calculations of % latent TGF-β (hatched bars) compared to % active TGF-β (shaded bars) were completed. Figure 5A demonstrates the mean activity of the TGF-β in the samples collected at the 24 hour time point, Figure 5B -- the mean activity at 48 hrs.

A


% total TGF-β
100
80
60
40
20
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

B

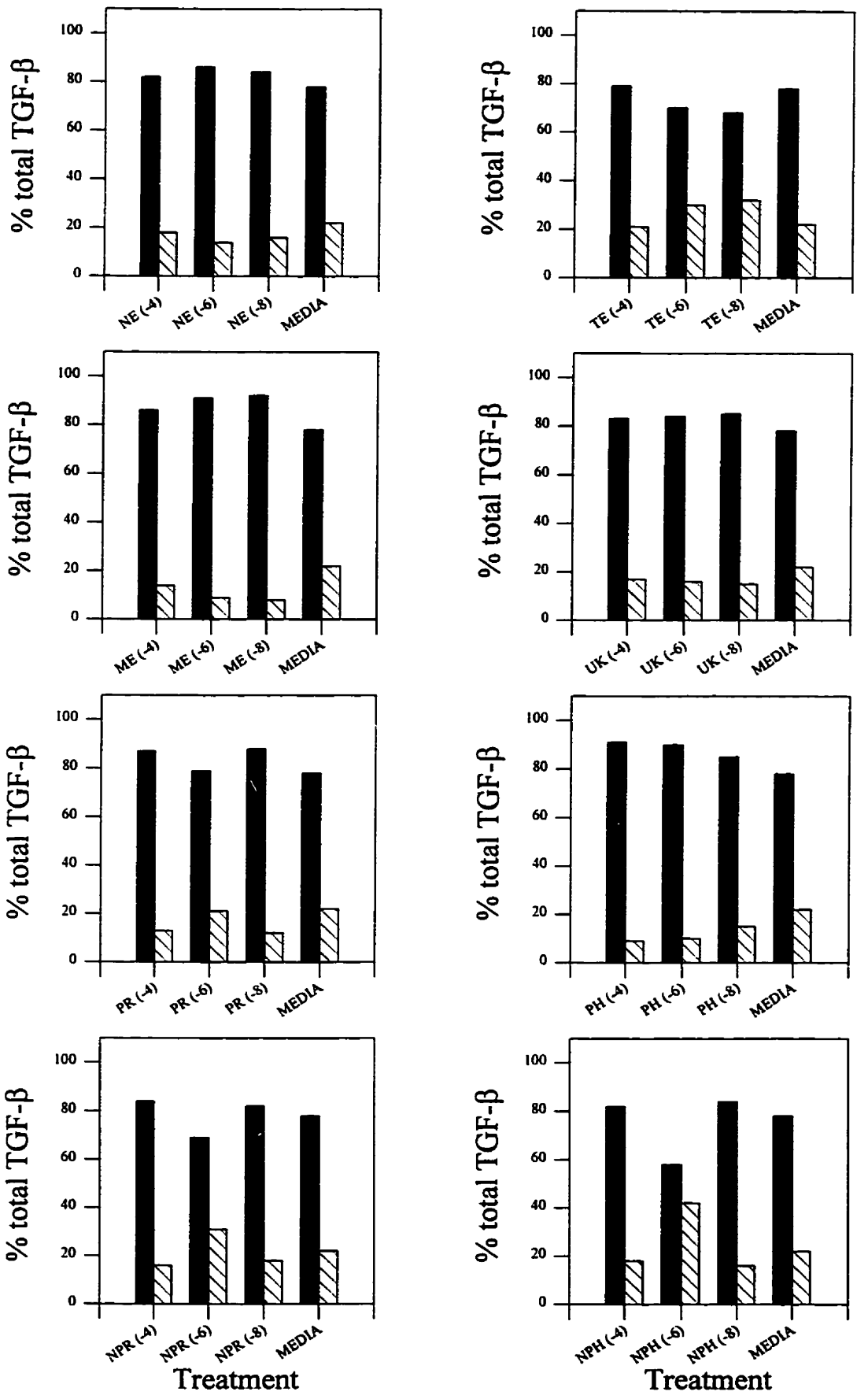
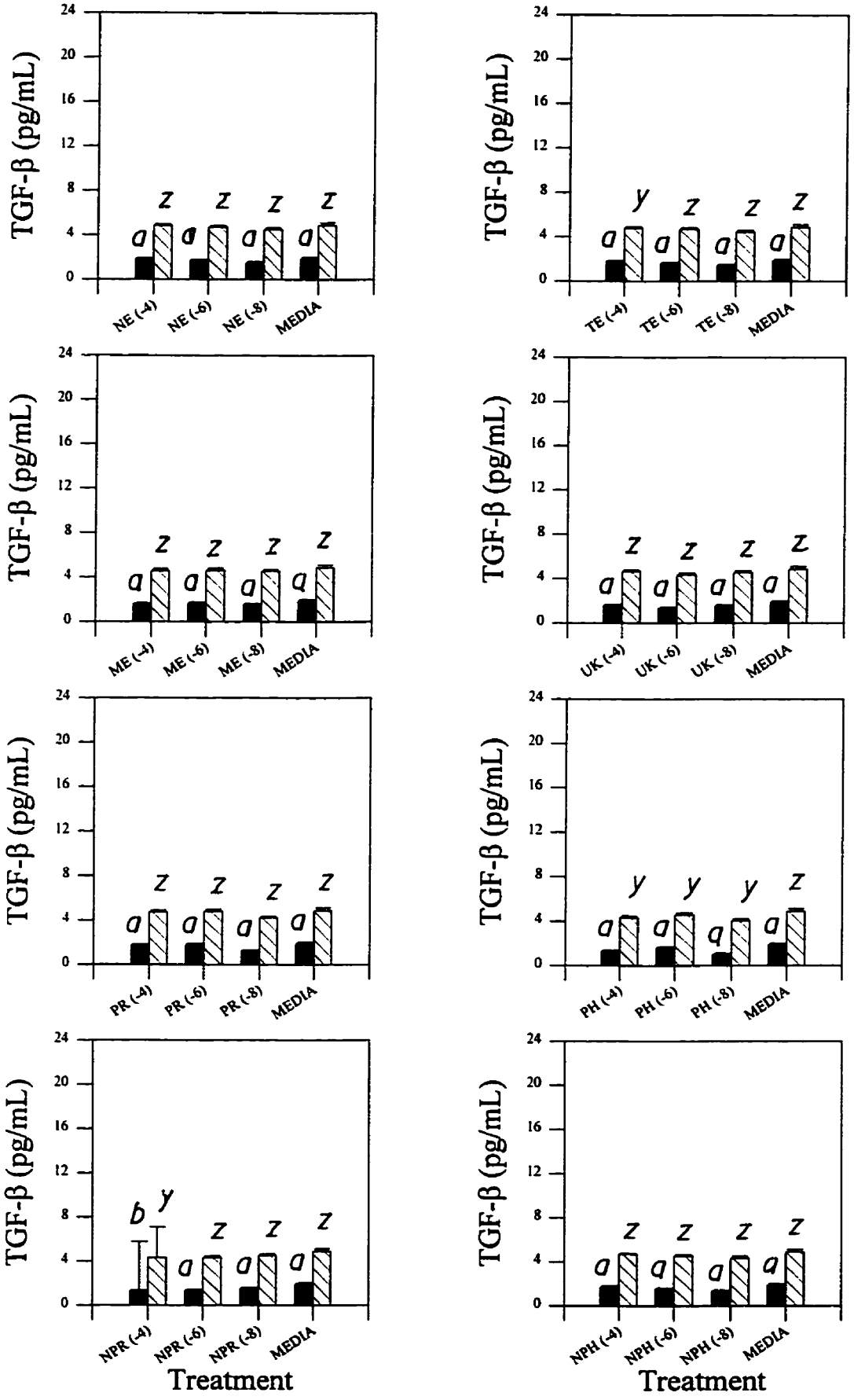

% total TGF-β
100
80
60
40
20
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

FIGURE 10. A comparison of mean total TGF-β production over a two day time course by non-stimulated murine splenocytes treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the total TGF-β production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	PR	≤0.001	≤0.001
TE	≤0.001	≤0.001	PH	≤0.001	≤0.001
ME	≤0.001	≤0.014	NPR	≤0.015	≤0.019
UK	≤0.001	≤0.001	NPH	≤0.001	≤0.001


TGF-β (pg/mL)
24
20
16
12
8
4
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

β. In each of these figures, the 24 hr data is illustrated with solid bars, while the 48 hr data is represented by the hatched bars.

All of the various adrenergic agonist and antagonist treatments had effects on the production of total TGF-β at 24 and 48 hrs. It is however, illustrated that NE had a significant effect on the production of total TGF-β by non-stimulated murine splenocyte populations over the two time course points, and the results also display that the individual α- and β-adrenergic treatments had the same α- and β-adrenoceptor specific effects previously observed in the transformed cell lines -- with stimulation of either adrenoceptor resulting in an increase in total TGF-β production. Although many of the adrenergic treatments had significant effects on total TGF-β production over both time course points, these increases in total TGF-β are not tremendous.

The adrenergic agonist and antagonist treatments appear to have had no significant effect on the production of active TGF-β by the non-stimulated murine splenocytes over the two time course points (Figure 11), although significant increases in active TGF-β production were observed for the UK and NPH treatments.

Evaluating the above data for total TNF-α production by non-stimulated murine splenocytes over the two day time period using Tukey's pairwise comparison illustrates that there are significant differences between the media control treatment and some adrenergic treatments, but not others. (Figure 10 and Figure 11, respectively). The addition of anti-TGF-β_1 antibodies to duplicate samples of conditioned media demonstrated that the effects illustrated throughout the experiments were due to TGF-β_1 and thus the A375 assay was able to measure the quantities of TGF-β secreted by the murine splenocytes.

Effects of adrenergic agonist and antagonist treatments on the production of biologically latent and active TGF-β by non-stimulated murine splenocytes. TGF-β was again quantified both before and after activation by acidification to measure %

FIGURE 11. A comparison of mean active TGF-β production over a two day time course by non-stimulated murine splenocytes treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the active TGF-β production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.799	≤0.945	PR	≤0.857	≤0.874
TE	≤0.819	≤0.842	PH	≤0.814	≤0.947
ME	≤0.815	≤0.998	NPR	≤0.846	≤0.952
UK	≤0.927	≤0.814	NPH	≤0.558	≤0.870

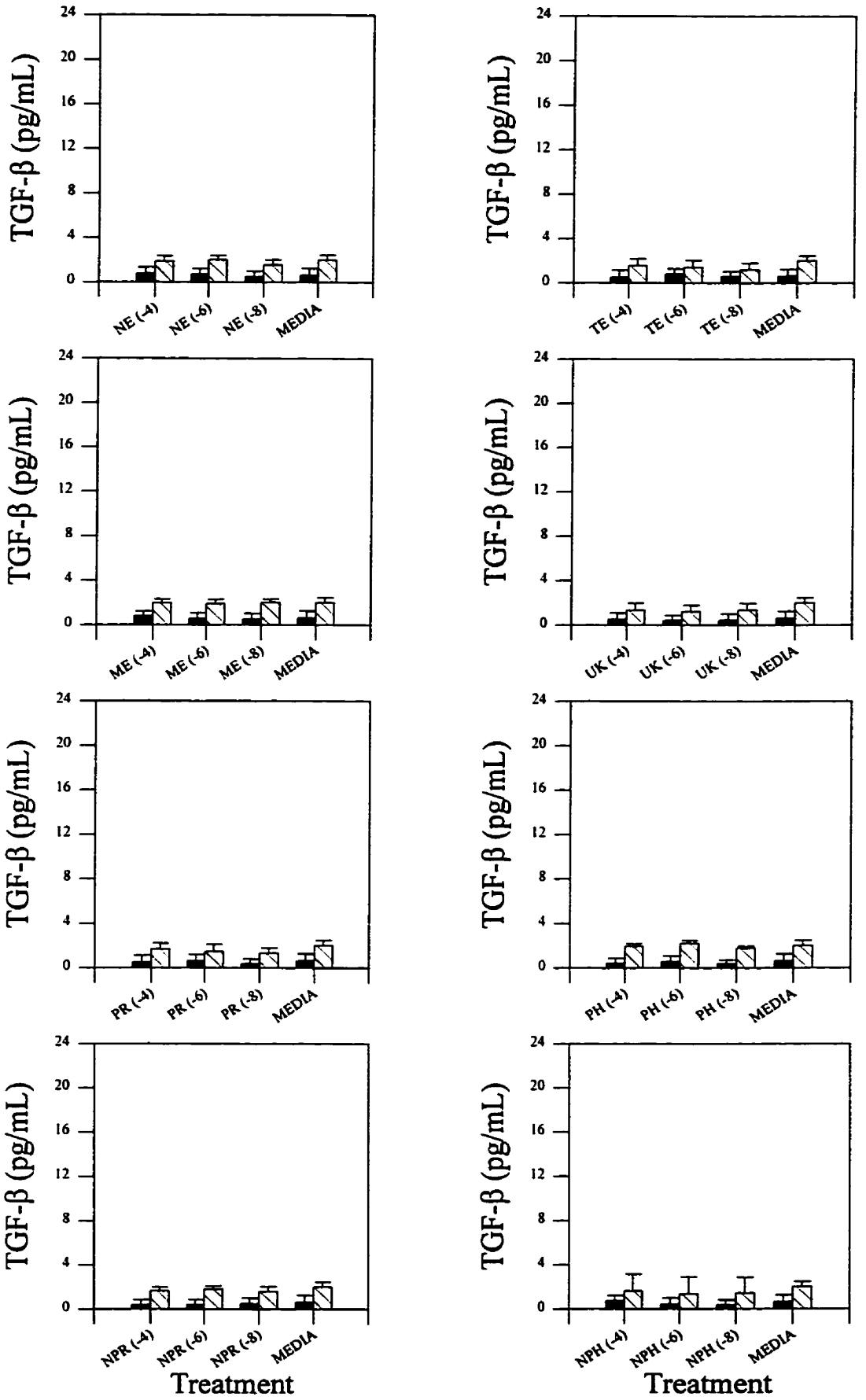
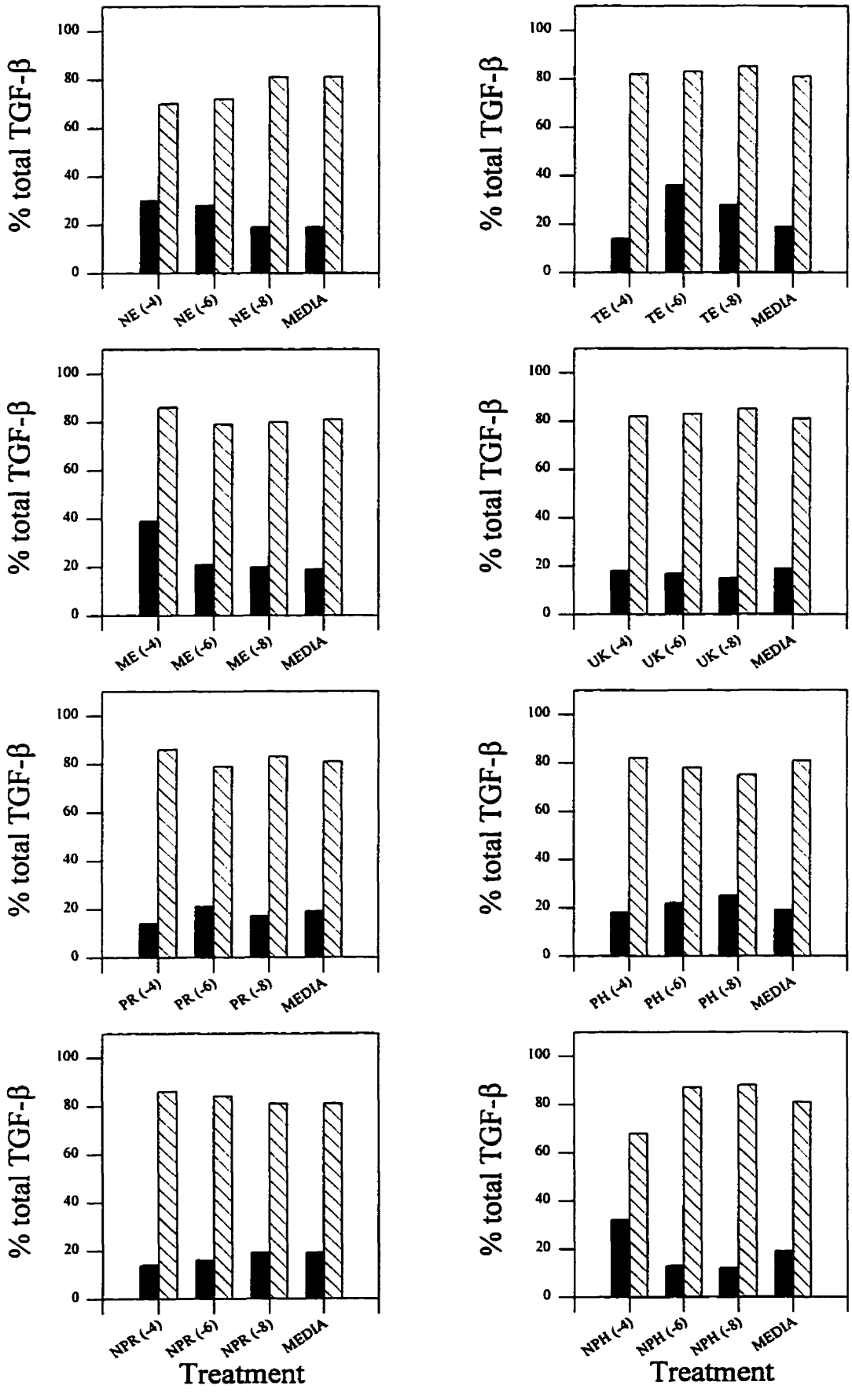
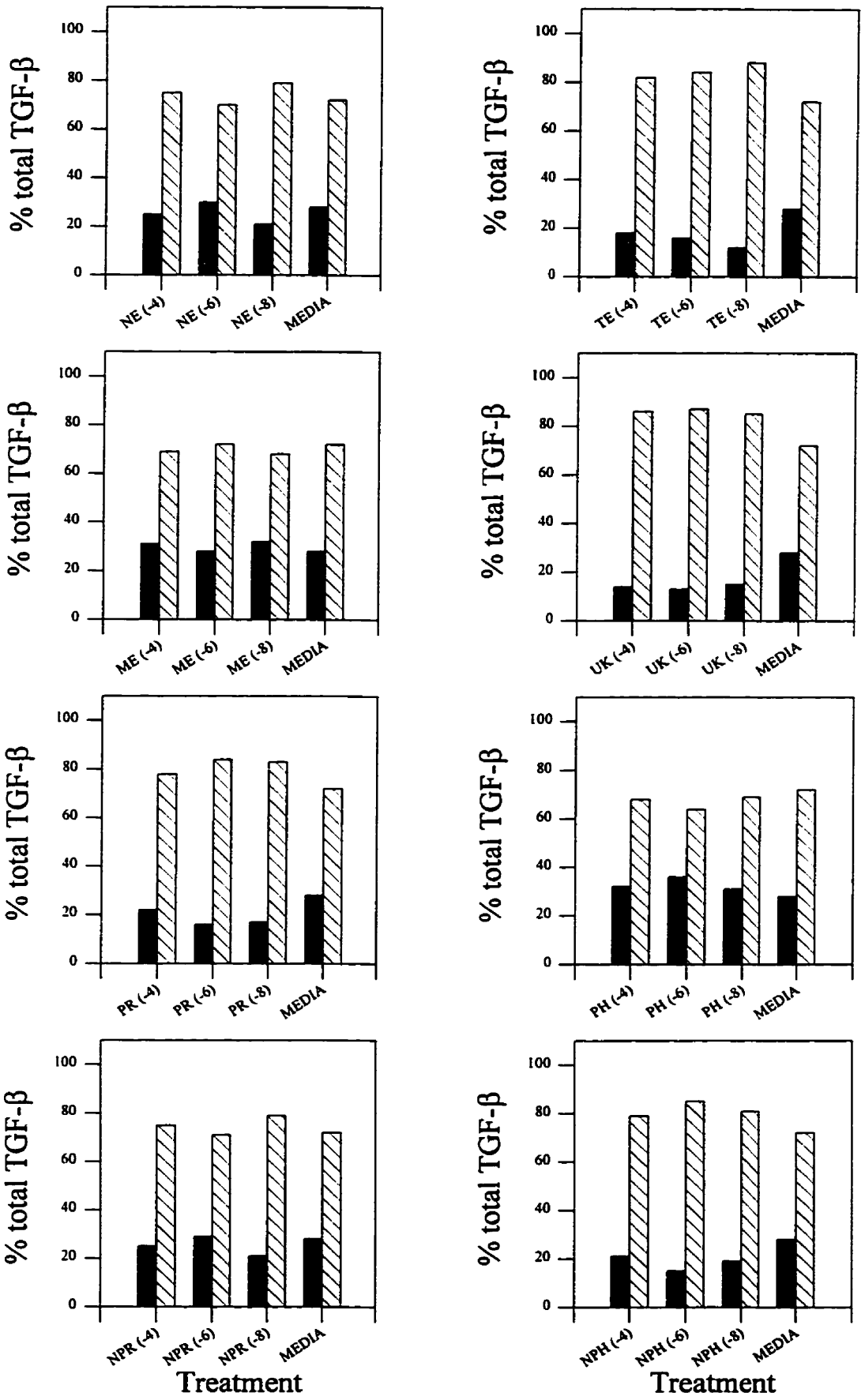

TGF-β (pg/mL)
24
20
16
12
8
4
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

FIGURE 12. The biological activity of the TGF-β produced by non-activated murine splenocytes treated with various adrenergic agonists and antagoinsts. Based on the total TGF-β concentrations obtained through experimentation (Figure 9), calculations of % latent TGF-β (hatched bars) compared to % active TGF-β (shaded bars) were completed. Figure 5A demonstrates the mean activity of the TGF-β in the samples collected at the 24 hour time point, Figure 5B -- the mean activity at 48 hrs.

A


% total TGF-β
100
80
60
40
20
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

B


% total TGF-β
100
80
60
40
20
0
NE (-4)
NE (-6)
NE (-8)
MEDIA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

latent versus % active TGF-β. The results of this acidification process of the total TGF-β obtained in Figures 9 are observed in Figure 12 -- A and B for the 24 and 48 hr time course respectively, as % latent TGF-β (shaded bars) versus % active TGF-β (black bars). It is interesting to note that predominantly all TGF-β secreted by the non-activated murine splenocytes was in a latent form.

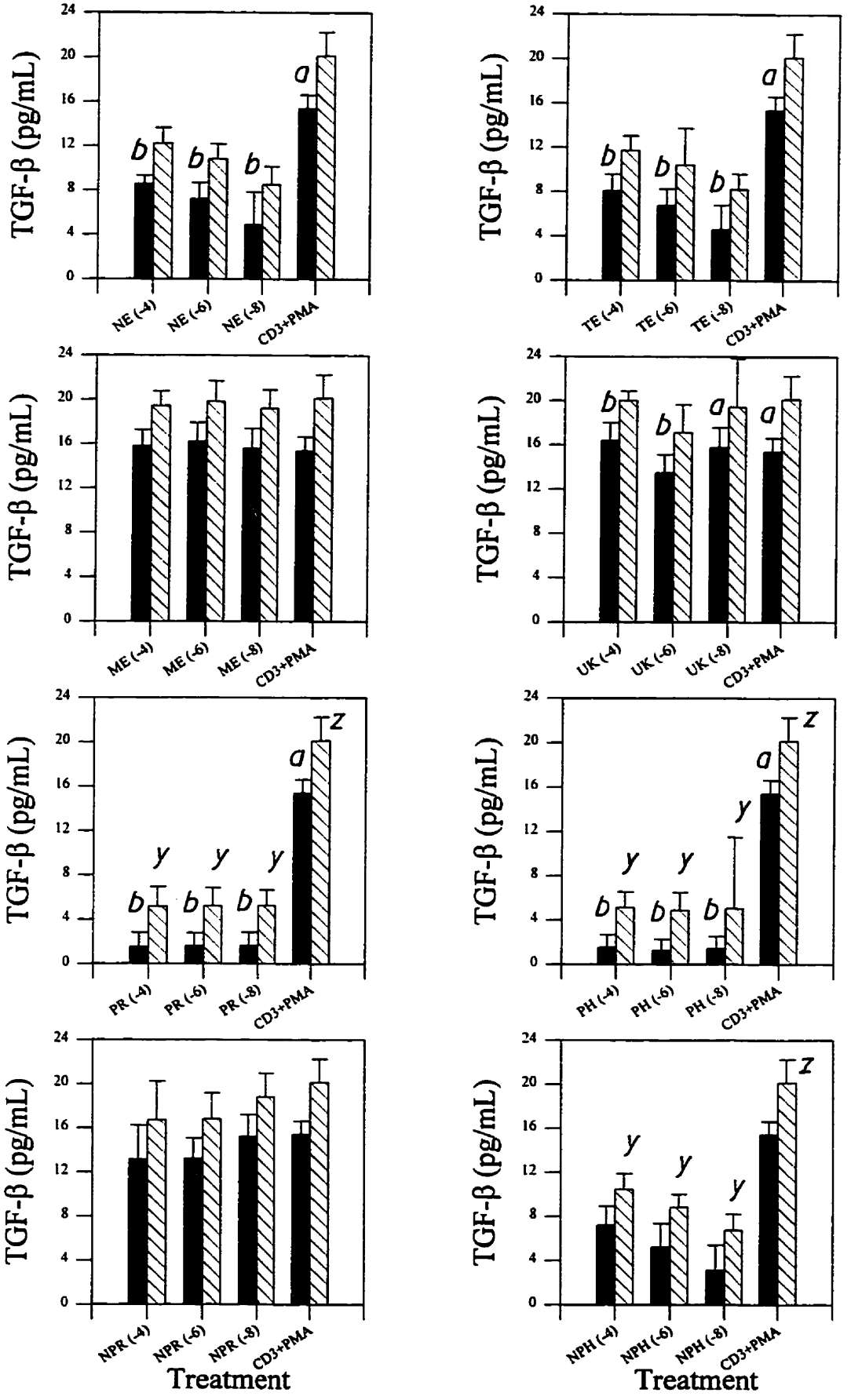
Adrenergic Agonist and Antagonist Treatments, CD3 + PMA Stimulated Murine Splenocytes and Transforming Growth Factor-β

The production of TGF-β by CD3 + PMA stimulated murine splenocytes in response to the adrenergic agonist and antagonist treatments was also examined over the concentration range of 10^{-4}, 10^{-6}, 10^{-8} M. Samples of the conditioned media were collected over a two day time course and assayed for TGF-β. The results of these experiments are displayed in Figure 13 for total TGF-β and Figure 14 for active TGF-β. In each of these figures, the 24 hr data is illustrated with solid bars, while the 48 hr data is represented by the hatched bars.

From preliminary observations, it can be concluded that NE had a significant effect on modulating the production of total TGF-β by CD3 + PMA stimulated lymphocytes at the 24 hr time interval as the production of total TGF-β was significantly inhibited when the splenocytes were co-cultured with NE and CD3 + PMA. The results utilizing the α-adrenergic agonists ME and UK, illustrate that neither α-agonist had a conclusive, significant effect on total TGF-β production. In the CD3 + PMA stimulated splenocytes treated with NPR, NE was able to target only the α-adrenoceptor to modulate TGF-β production. From these results, NPR treatments in conjunction with the CD3 + PMA stimulated murine splenocytes, it is observable that there was no significant increase in total TGF-β production. Thus, from the results obtained utilizing ME, UK and NPR, it can be concluded that α-adrenoceptor modulation did not significantly increase total TGF-β production.

FIGURE 13. A comparison of mean total TGF-β production over a two day time course by CD3 + PMA stimulated murine splenocytes treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the total TGF-β production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.110	PR	≤0.001	≤0.006
TE	≤0.001	≤0.074	PH	≤0.001	≤0.004
ME	≤0.141	≤0.814	NPR	≤0.138	≤0.203
UK	≤0.014	≤0.697	NPH	≤0.138	≤0.049


TGF-β (pg/mL)
NE (-4)
NE (-6)
NE (-8)
CD3+PMA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

Ensuing experiments examining the effect of β-adrenoceptor modulation on TGF-β production by CD3 + PMA stimulated splenocytes illustrated that the β_2-adrenergic agonist TE did not have consistent, significant inhibitory effects. The effect of β-adrenoceptor inhibition on total TGF-β production was however observed when NE was added in combination with PH (NPH). From these mixed results utilizing the TE and NPH treatments, it is difficult to conclusively determine if NE can target the β-adrenoceptor of the CD3 + PMA stimulated splenocyte to inhibit total TGF-β production.

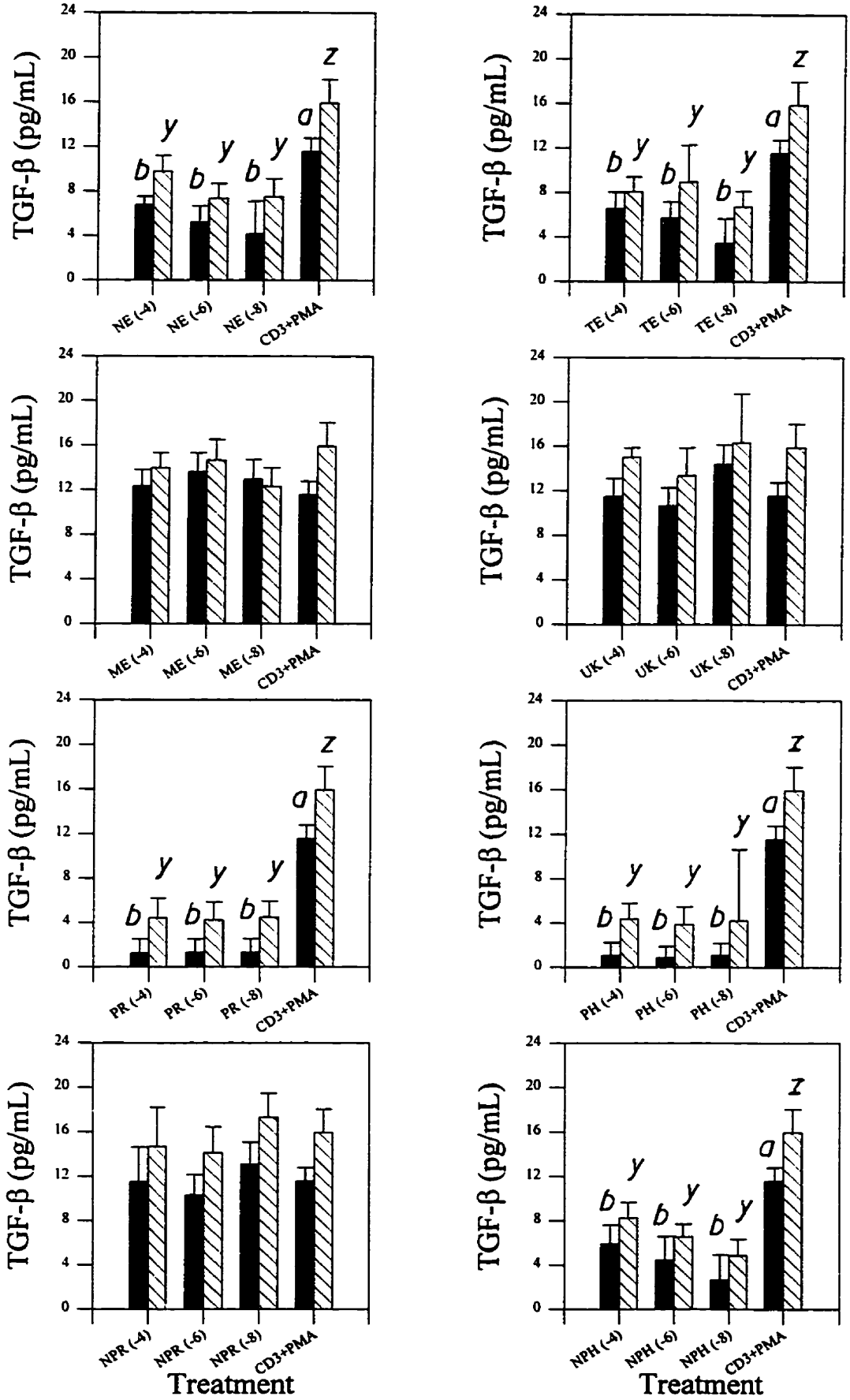
In conclusion, the relationship between NE modulation and TGF-β production is difficult to determine. Although, TGF-β production was decreased by some adrenergic treatments over some of the time course points, no consistent effects are observed and thus a complete, interpretable determination of the effects of NE in modulating total TGF-β production by CD3 + PMA stimulated murine splenocytes is impossible.

Examining the results of the various adrenergic treatments on active TGF-β production by the CD3 + PMA stimulated murine splenocytes over the two time course points (Figure 14), again illustrates that many of the adrenergic drugs did not have consistent or conclusive effects on the modulation of active TGF-β production. The treatments of NE, TE, PR, PH and NPH were all able to significantly inhibit the production of active TGF-β at 24 and 48 hrs, suggesting that β-adrenoceptor and not α-adrenoceptor modulation has an affect on TGF-β production by CD3 + PMA stimulate murine splenocytes.

Evaluating the above data for total TNF-α production by CD3 + PMA stimulated murine splenocytes over the two day time period using Tukey's pairwise comparison illustrates that there are significant differences between the media control treatment and some adrenergic treatments, but not others. (Figure 13 and Figure 14, respectively). The addition of anti-TGF-β_1 antibodies to duplicate samples of

FIGURE 14. A comparison of mean active TGF-β production over a two day time course by CD3 + PMA stimulated murine splenocytes treated with various adrenergic agonists and antagonists. Utilizing ANOVA, the means of the active TGF-β production for the experiment at 24 hrs (black bars) and 48 hrs (hatched bars) were statistically analyzed for signficance; an experimental size of n=6 was used to obtain each time course point. In each panel, the standard deviation of the experimental means is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	PR	≤0.001	≤0.001
TE	≤0.001	≤0.001	PH	≤0.001	≤0.001
ME	≤0.806	≤0.591	NPR	≤0.520	≤0.550
UK	≤0.324	≤0.610	NPH	≤0.001	≤0.001


TGF-β (pg/mL)
NE (-4)
NE (-6)
NE (-8)
CD3+PMA
TE (-4)
TE (-6)
TE (-8)
ME (-4)
ME (-6)
ME (-8)
UK (-4)
UK (-6)
UK (-8)
PR (-4)
PR (-6)
PR (-8)
PH (-4)
PH (-6)
PH (-8)
NPR (-4)
NPR (-6)
NPR (-8)
NPH (-4)
NPH (-6)
NPH (-8)
Treatment

conditioned media demonstrated that the effects illustrated throughout the experiments were due to TGF-β_1 and thus the A375 assay was able to measure the quantities of TGF-β secreted by the CD3 + PMA stimulated murine splenocytes (119, 128, 143).

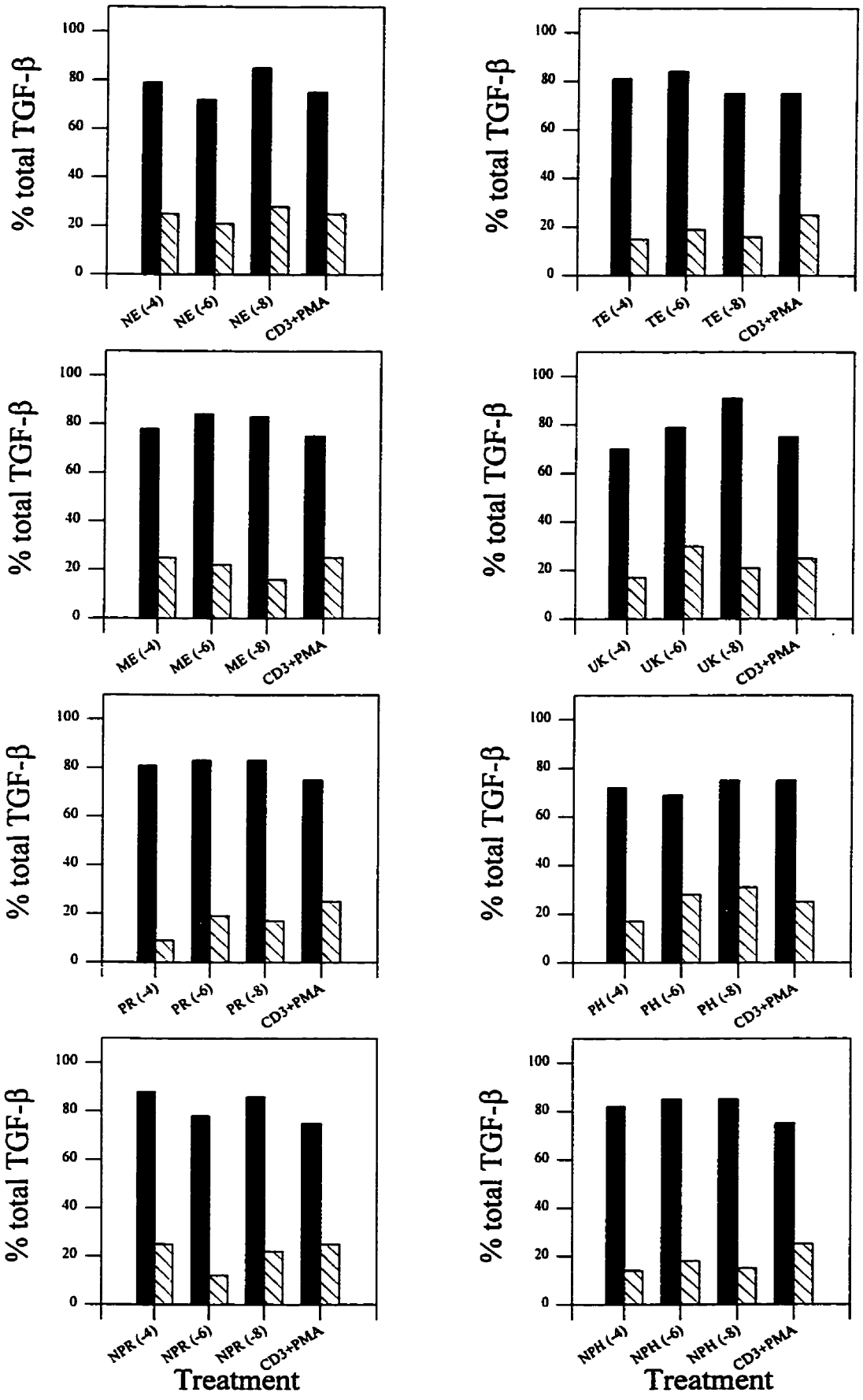
Effects of adrenergic agonist and antagonist treatments on the production of biological latent and active TGF-β by CD3 + PMA stimulated murine splenocytes. Total TGF-β was again quantified both before and after activation by acidification. The results of this acidification process of the total TGF-β obtained in Figures 13 can be observed in Figure 15 -- A and B for the 24 and 48 hr time course respectively, as % latent TGF-β (shaded bars) versus % active TGF-β (black bars). It is again visible through the results that predominantly all TGF-β secreted by the activated murine splenocytes was in an active form.

DISCUSSION

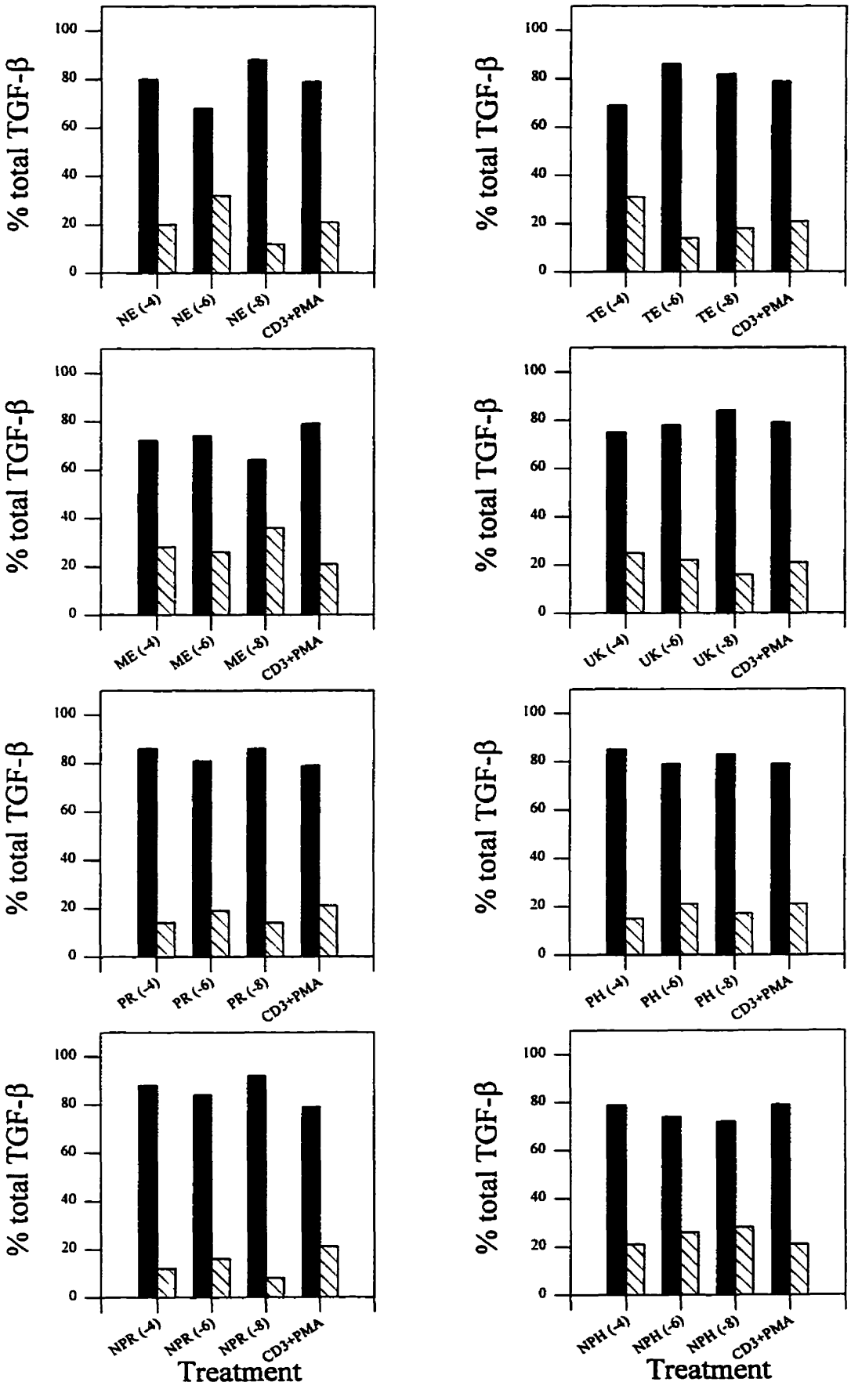
In previously reported studies involving NE and murine splenic macrophages, it was reported that NE was able to both increase and decrease the levels of total TGF-β and TNF-α. Because norepinephrine is both an α- and β-adrenergic agonist, it is able to modulate a variety of immunological effects through both the α- and β-adrenoceptors found on most lymphocytes. These findings, which demonstrate that norepinephrine partakes in immune cell modulation, are significant because they illustrate that not only does NE have a role in modulating cytokine production, but perhaps more interestingly -- the modulatory effects of norepinephrine are dependent upon the adrenoceptor target.

FIGURE 15. The biological activity of the TGF-β produced by CD3 + PMA stimulated murine splenocytes treated with various adrenergic agonists and antagoinsts. Based on the total TGF-β concentrations obtained through experimentation (Figure 12), calculations of % latent TGF-β (hatched bars) compared to % active TGF-β (shaded bars) were completed. Figure 5A demonstrates the mean activity of the TGF-β in the samples collected at the 24 hour time point, Figure 5B -- the mean activity at 48 hrs.

A


% total TGF-β
100
80
60
40
20
0
NE (-4)
NE (-6)
NE (-8)
CD3+PMA
TE (-4)
TE (-6)
TE (-8)
CD3+PMA
ME (-4)
ME (-6)
ME (-8)
CD3+PMA
UK (-4)
UK (-6)
UK (-8)
CD3+PMA
PR (-4)
PR (-6)
PR (-8)
CD3+PMA
PH (-4)
PH (-6)
PH (-8)
CD3+PMA
NPR (-4)
NPR (-6)
NPR (-8)
CD3+PMA
NPH (-4)
NPH (-6)
NPH (-8)
CD3+PMA
Treatment
Treatment

B


% total TGF-β
100
80
60
40
20
0
NE (-4)
NE (-6)
NE (-8)
CD3+PMA
TE (-4)
TE (-6)
TE (-8)
CD3+PMA
ME (-4)
ME (-6)
ME (-8)
CD3+PMA
UK (-4)
UK (-6)
UK (-8)
CD3+PMA
PR (-4)
PR (-6)
PR (-8)
CD3+PMA
PH (-4)
PH (-6)
PH (-8)
CD3+PMA
NPR (-4)
NPR (-6)
NPR (-8)
CD3+PMA
NPH (-4)
NPH (-6)
NPH (-8)
CD3+PMA
Treatment
Treatment

The β-adrenoceptor, Norepinephrine and Transforming Growth Factor-β Modulation

The modulatory effects of norepinephrine on the production of TGF-β were studied utilizing a variety of adrenergic drugs including norepinephrine and terbutaline. The results obtained illustrate that norepinephrine is able to significantly increase the production of total TGF-β by both transformed cell lines, EL-4s and Jurkats, as well as by the non-stimulated murine splenocyte population, while the CD3 + PMA stimulated murine splenocyte population produced significantly less TGF-β in the presence of norepinephrine. Since NE was found to significantly enhance the production of total TGF-β, and because it is known that norepinephrine is able to target both adrenoceptor subtypes, a study of the adrenoceptor through which NE was modulating it's effect was undertaken.

Subsequent studies utilizing the β_2-adrenergic agonist, terbutaline, illustrated similar effects to those observed with the NE treatments, notably, TE was able to significantly increase total TGF-β production to similar quantities in both populations of transformed T cells. Although this effect likely occurred because of TE's efficacy for the β_2-adrenoceptor, future studies will be needed to further characterize the β-adrenoceptor responsible for this response, since TE has affinity for both β_1 and β_2-adrenoceptors (50). Notably, the results of the treatments of NE and TE on these transformed cultures are similar in their magnitude of response as well their apparent dose dependent effects, thus it was concluded that NE modulates an increase in total TGF-β production through the β-adrenoceptor of the these cell lines.

In the non-stimulated murine splenocyte populations, the three concentrations of NE had an observable effect on total TGF-β production. In comparing the murine splenocyte populations treated with an adrenergic drugs to cells treated with only media, it was concluded that the adrenergic drug treatments had significant effects on total TGF-β production at 48 hrs and 72 hrs, but no effect at 24 hrs after commencing

incubation. TE was again able to elicit similar stimulatory increases in total TGF-β production as were seen with the NE treatments, and thus it was reasoned that the modulation of TGF-β by TE and NE was through the β-adrenoceptor of the non-stimulated murine splenocyte and was related to the concentration of the treatment.

When both the stimulatory treatment of CD3 + PMA and the adrenergic treatments were co-cultured with the murine splenocytes, NE and TE both significantly decreased total TGF-β production, and this inhibition additionally appears to be related to the concentration of the adrenergic drug. The effects of the NE and TE treatments were thus concluded to be mediated by β_2-adrenoceptor modulation since both TE and NE again had similar effects on total TGF-β production by the CD3 + PMA stimulated murine splenocyte population.

The α-Adrenoceptor, Norepinephrine and Transforming Growth Factor-β Modulation

Although it was concluded that NE could modulate the production of TGF-β by all four cell cultures through the β-adrenoceptor, norepinephrine is recognized for it's ability to bind to both the α- and β- adrenoceptor of lymphocytes. Because of this, an attempt was made to determine the effect of α-adrenoceptor modulation on total TGF-β production. To study the effect of NE on the α-adrenoceptor subtypes, studies were performed utilizing the α_1 and α_2-adrenergic agonists ME and UK, respectively. These experiments illustrate conclusively that ME and UK treatments were also able to significantly affect the production of total TGF-β by all four lymphocyte populations.

The Adrenergic Antagonists, Norepinephrine and Transforming Growth Factor-β Modulation

Concurrent to the experiments with NE, TE, ME or UK on the four populations of cells, experiments studying the effect of NE in the presence of adrenergic antagonists were also performed. PH, an α-adrenergic antagonist and PR, a β-adrenergic antagonist were co-cultured with NE in a 1:1 ratio at three different concentrations with each of the different cell types and again the collected supernatants were assayed for TGF-β. Because of their adrenoceptor blocking abilities, both PH and PR treatments should eliminate the effect of NE through the α- and β-adrenoceptor, respectively.

In the population of non-activated murine spleen cells, the NPR and NPH treatments had no observable differences in the production of total TGF-β production as compared to the other adrenergic treatments, although total TGF-β production was significant as compared to the media control. The murine splenocytes which received the CD3 + PMA stimulation and NPR displayed inconsistent effects -- insignificant increases in total TGF-β production as compared to the CD3 + PMA stimulated control population were observed, although the overall production of TGF-β was increased significantly as compared to the media control population. In the population of murine splenocytes treated with NPH in conjunction with CD3 + PMA, a significant decrease in TGF-β production was observed compared to the cells which received only the CD3 + PMA treatments, although as compared to the media control this production appears again significantly increased. Thus, from these results it is again observable that the outcome of NE modulation on TGF-β production depends upon which adrenoceptor is targeted by NE.

The experimental results of the NE treatments in the presence of either PR or PH display significant increases in total TGF-β by both EL-4 and Jurkat cultures. Although the production of TGF-β by the NPR treatment was greater than by the

NPH treatments, both of these treatments produced significant increases in total TGF-β production, thus demonstrating that even when the α- and β-adrenoceptors were blocked with NPH and NPR, respectively, NE was still able to modulate a response.

The results which were obtained through the use of the antagonist treatments alone, also present interesting concepts in relation to the modulation of TGF-β production by NE. The addition of the treatments of PH and PR both produced significant increases in TGF-β production by the EL-4 and Jurkat cell lines as well as by the CD3 + PMA stimulated and non-stimulated murine splenocyte populations. The treatments of the antagonist blockers were meant originally as a control to determine the individual effects of PH and PR on TGF-β production by the cell cultures. However, in the results, it appears that either they were individually able to mediate the production of total TGF-β, or perhaps each of the studied cell types was able to secrete endogenous NE. Thus, it remains plausible that if the cells were releasing endogenous stores of NE, and the cells were subsequently treated with an adrenergic blockers, that the effect of the endogenous NE would be mediated through the receptor which was not blocked by an antagonist. This final hypothesis of modulation of cytokine production by endogenous norepinephrine will need to be examined in future research

The Biological Activity of Transforming Growth Factor-β and Isoform Identification

Because TGF-β is produced in two biological forms and many isoforms, it was imperative to determine both the effects of the adrenergic treatments on % latent versus % active TGF-β and on TGF-β isoform secretion. From the experimental results, it is indicated that predominantly all TGF-β produced by the Jurkats, EL-4s and CD3 + PMA stimulated murine splenocytes was released in an active form, while the TGF-β released by the non-stimulated murine splenocytes was in a latent form.

The significance of these findings are potentially important to both disease process and disease outcome.

TGF-β is only biologically active when it has been activated by proteolysis, pH or by the many unknown mechanisms involved in tumor development (108, 111, 119, 120, 126). Thus it appears that the secretion of latent TGF-β by cells is an important regulatory mechanism since most cells ubiquitously express both TGF-β and it's receptors. In this research project, the increased secretion of TGF-β in an active form may be of clinical importance, since in it's active form, TGF-β is involved in the regulation of many immune responses, including the fibrosis reaction in Idiopathic Pulmonary Fibrosis.

The isoform secreted by these four cell types was found to be of the TGF-β_1 isotype through the use of anti-transforming growth factor-β_1 antibodies. Most of the TGF-β present in the samples was neutralized by this isoform; reviews of the literature on TGF-β isoforms suggest that this is what is expected (128, 143, 149, 151). There are three known isoforms of TGF-β found in mammalian cells, however, the biological activities of these isoforms in not distinguishable in most *in vitro* assays (151). Although very little information is known about the mechanism of differential expression of TGF-β isoforms, it has been shown that each isoform is regulated by a different mRNA promoter (151). Research has indicated that TGF-β_1 is predominately secreted by non-stimulated murine macrophages with lesser amounts of TGF-β_2 and TGF-β_3 present. It has also been found that increased quantities of TGF-β_1 are secreted by activated murine macrophages, however, TGF-β_2 and TGF-β_3 amounts remain relatively unchanged. Studies examining T lymphocytes activated by PMA in the presence of corticosteroids have also been shown to have an increase in secretion of TGF-β_1 quantities. Thus, the results of this study relating to the isoform of TGF-β present in the samples, TGF-β_1, appears to agree with published literature (151).

The Complex Role of Norepinephrine in Transforming Growth Factor-β Modulation by Transformed and Primary Cell Cultures

Current literature has hypothesized that T helper 1 and T helper 2 cells differentially express the β-adrenoceptor, and that the expression of this receptor does not differ with activation (99, 100, 104, 105). It has also been hypothesized through research that the β_2-adrenoceptor of activated Th1 and Th2 cells differentially modulates the production of cytokines as compared to non-activated Th1 and Th2 cells (99, 100, 104, 105). Scientists have demonstrated that exposure of activated Th2 cells subsets to β_2-adrenoceptor agonists has no effect on inducing Th2 cytokine production, although exposure of activated Th1 cells to β_2-adrenoceptors decreases the production of many cytokines (99, 100, 104, 105). These findings therefore can be interpreted to propose that differential expression of the β_2-adrenoceptor on activated Th1 and Th2 clones renders cytokine production by these clones differentially susceptible to the influence of the β_2-adrenoceptor ligand (99, 100, 104, 105). Although tremendous amounts of published work have studied the β-adrenoceptor found on lymphocyte populations, very little research has focused on the potential presence of an α-adrenoceptor (99, 100, 104, 105). Although several studies have suggested that α-adrenergic agonists can modify lymphocyte function, α-adrenoceptors as yet remain to be classified by ligand binding studies and therefore their possible existence remains a controversial issue in neuroimmunology (99, 100, 104, 105).

The transformed populations, EL-4s and Jurkats presented a very distinct response to the addition of the adrenergic drug treatments. NE increased TGF-β production through the β_2-adrenoceptor, although the increase was not as great as was seen when the stimulus was only through the α-adrenoceptor. Although this result is contradictory to the published studies relating to the decreased production of cytokines by T cells stimulated with β_2-adrenoceptor agonists, no research has been

performed on characterizing the production of TGF-β by T lymphocyte neuroimmunomodulation (99, 100, 104, 105). Also since both of these cell lines did not receive an activation signal, there remains a question as to the possibility of NE mediating a direct effect on T cell production of TGF-β by these transformed cell lines.

The results obtained in this research project also suggest that the murine splenocyte population which was stimulated with CD3 + PMA exhibited a β_2-adrenoceptor effect when NE was added since the production of the studied cytokine, TGF-β, was similarly decreased in the presence of TE, a β_2-adrenoceptor agonist. This is what is expected in a Th1 response since it is known, as previously stated, that activated Th1 cells exposed to a β_2-adrenoceptor ligand will decrease production of IL-2 (99, 100, 104, 105). Although an inhibitory response (Th1 response) to the presence of β_2-adrenoceptors occurred in the CD3 + PMA stimulated population of murine splenocytes, this response was absent in the experiments performed with NE and non-stimulated splenocyte populations. Without further experimental probing, the reasoning for these differences in conclusions between this research project and those previously published are unknown. Some of the differences may be due to the mixed population of B cells, macrophages and T cells in the non-stimulated murine splenocyte population and therefore the data may reflect a conglomerate of responses to norepinephrine; responses that are individually in-distinguishable. Since there was no clear activation signal for any of the lymphocyte populations in the non-stimulated murine splenocyte samples, a mixed response may have occurred and TGF-β production by the mixed cell population is not representative of NE modulatory effects on T cell populations alone.

Finally, research has examined the presence of endogenous stores of NE in macrophage cells (177), however, no research has been published regarding the same phenomenon in lymphocyte populations. In the macrophage studies involving the endogenous stores of NE, it was concluded that the β-adrenergic antagonist

propranolol was able to augment the production of TNF, however, the α-adrenergic treatments of yohimbine and idazoxan had an inhibitory effect on TNF-α production (177). These results, therefore, suggest evidence for the extraneuronal accumulation of norepinephrine, however they do not define the physiological reasoning for this system (177). Thus, it is imperative that this hypothesis of endogenous norepinephrine be examined in the lymphocyte populations as it may further both the ideas of neuroimmunomodulation of cytokine production as well as support the possibility that norepinephrine can be released endogenously by the immune system to regulate immune responses.

Conclusions Regarding Transforming Growth Factor-β Modulation by Norepinephrine

In summary, the results of this research project propose that norepinephrine, a sympathetic nervous system neurotransmitter, can increase the production of TGF-β of two transformed cell lines and non-activated murine splenocytes through both α- and β-adrenergic agonist treatments, with the α-adrenergic agonist treatment being a greater stimulant of TGF-β production. It was also concluded that norepinephrine was also able to affect TGF-β production by CD3 + PMA activated murine splenocytes through the β-adrenoceptor, and that this inhibition is most marked at submaximal concentrations of the catecholamine.

CHAPTER IV:

THE MODULATION OF TUMOR NECROSIS FACTOR-α AND TRANSFORMING GROWTH FACTOR-β BY NOREPINEPHRINE IN LIPOPOLYSACCHARIDE STIMULATED AND NON-STIMULATED MURINE ALVEOLAR MACROPHAGES

Significant progress has been made in determining that a variety of cytokines play valuable roles in pulmonary fibrosis, and current research continues to demonstrate the effects that the nervous system has on cytokine production. This chapter focuses on the possible interconnections between the immune and nervous systems within the respiratory system. Bronchoalveolar lavage was performed on six mice to collect alveolar macrophage samples. Alveolar macrophages were subsequently stimulated with LPS and adrenergic agonists and/or antagonists to determine the effect of norepinephrine on the production of transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α). In this study, norepinephrine significantly influenced LPS-stimulated alveolar macrophage production of both TGF-β and TNF-α over a two day time course. The production of TGF-β was modulated by norepinephrine through both the α- and β-adrenoceptors of the LPS stimulated murine alveolar macrophages. Stimulating the α-adrenoceptor with norepinephrine in combination with propranolol (NPR) resulted in an increased total TGF-β production, while a decrease in total TGF-β production resulted when the LPS stimulated alveolar macrophages were treated only with norepinephrine. A decrease in total TGF-β production was also observed when the β-adrenoceptor was stimulated with terbutaline or a combination of norepinephrine and phentolamine (NPH). The biological activity of the TGF-β secreted by the alveolar macrophages suggests that LPS stimulation results in the production of active TGF-β, while a lack of LPS stimulation relates to increases in latent TGF-β production. The production of TNF-α by LPS stimulated murine

alveolar macrophages was similarly decreased in the presence of norepinephrine and also when norepinephrine was combined with phentolamine (NPH). These results suggest the modulation of TNF-α and TGF-β by norepinephrine through the β-adrenoceptors of alveolar macrophages.

INTRODUCTION

Within the fluid-lined epithelial surfaces of the respiratory tract lies a tremendous number of immune cells, including alveolar macrophages (81, 146, 147, 148). Since the discovery in 1961 of a harvest method for these lung cells through bronchoalveolar lavage, alveolar macrophages have been extensively investigated (81, 147, 148). Alveolar macrophages account for upto 95% of cells recovered by lavage in most species tested to date (81, 146, 147, 148, 149, 151). A significant number of immunocompetent lymphocytes, predominantly T cells, are present in the lavage fluid, and often low numbers of eosinophils and polymorphs can also be retrieved (26, 81, 146, 147, 149). Although the respiratory system is constantly coming into contact with inspired antigens, as well as self antigens, the methods which these cells utilize to deal both with foreign particulate and microorganisms remains to be defined (81, 146, 147, 149).

The Alveolar Macrophage

Alveolar macrophages are representative of large tissue macrophages with a well developed vacuolar apparatus and a centrally located nucleus (26, 57, 81, 149, 150). Their origin is much debated, however, a consensus suggests that only two possible cell sources exist (81, 148). One hypothesis considers the cells arising from peripheral blood monocytes that migrate into the lung, and the second idea implicates the development of local mononuclear phagocytes that replicate in the alveolus or interstitium (20, 57, 81, 146, 147, 150). At the end of their lifespan, 21-28 days,

alveolar macrophage populations have been noted to move either into the lymphatics, or cross the alveolar epithelium and join the interstitium (116, 147).

Classically, the macrophage has been viewed chiefly as a phagocytic cell, and in the lung, ingestion of inhaled inorganic molecules and microorganisms is one of the most important functions of the alveolar macrophage (26, 81, 146, 147, 152). This role of the alveolar macrophage, as a phagocyte, has been recorded in numerous studies (146, 147, 148, 149). These published experiments illustrate that the alveolar macrophage is capable of phagocytosing massive numbers of microorganisms. In fact, the other known mechanisms of pulmonary defense such as clearance, utilizing the mucocililary elevator, are relatively limited in immune defense as compared to the alveolar macrophage (81, 146, 148, 149, 153).

In response to immunologic stimuli, macrophages and lymphocytes react through a complex series of stimulatory and inhibitory signals (146, 149). With the exception of certain antigen-specific B cell responses, lymphocyte activation is dependent upon the release of mediators by macrophages and macrophage activation in turn, often depends on the secretion of specific lymphokines (146, 149). Following antigen presentation or stimulation with factors, including lipopolysaccharide, macrophages produce interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) (57, 157). It is well established that this IL-1 has many functions, including a large number of non-lymphocyte target cells, triggering systemic defense reactions to infection like fever, increased hepatic production of acute phase proteins, and neutrophil recruitment (57, 146, 149). The alveolar macrophage is also able to secrete a wide variety of other cytokines, and recently much research has focused on the presence of abnormal levels of these cytokines in specific lung diseases, such as chronic lung disease of prematurity, sarcoidosis and IPF (140, 146, 149, 153).

The complex role of the alveolar macrophage in mediating the immune response allows this cell type to regulate the remodeling and repair process of the lung (78, 81, 149, 152). For example, there is increasing evidence to suggest that in the normal lung, alveolar macrophages release a factor which inhibits fibroblast growth (149). This unnamed factor has been demonstrated to increase fibroblast production of PGE2, a known suppressor of fibroblast proliferation (57, 149, 157). In diseases of the lung that are characterized by pulmonary fibrosis, alveolar macrophages are known to secrete at least two mediators that when combined promote fibroblast recruitment, attachment and proliferation (149). Normal macrophages produce low levels of the first of these factors, fibronectin, and it has been demonstrated that this factor enhances the binding of particulate matter, especially invading microorganisms (149).

Alveolar Macrophages, Cytokines, and Pulmonary Fibrosis

Pulmonary fibrosis is an irreversible accumulation of connective tissue in the interstitium of the lungs (43, 57, 81, 143, 151, 154, 155, 156, 161). Although research on animal models and studies of human lung fibrosis suggest the initiating events of this disease are due to a combination of pulmonary injury and the recruitment of macrophages to deal with the resulting inflammation -- the pathogenesis of pulmonary fibrosis is not well understood (109, 143, 154, 155, 156, 157, 158). A number of well characterized cytokines, including TGF-β, have been found in the injured lung, and/or have been found to be produced by inflammatory cells removed from the lungs of these patients (81, 138, 143, 150, 154, 156, 157, 158, 159). In an animal model, studied by Khalil and Greenberg (158), results indicated that the increased levels of TGF-β were the result of alveolar macrophages.

In the past several years, significant progress has been made in many aspects of pulmonary fibrosis research (42, 143, 156, 158, 160, 161). More specifically, scientists have found that a variety of cytokines, like transforming growth factor-β and

tumor necrosis factor-α, play important roles in the complex process of fibrosis (42, 81, 143, 156, 157, 159, 161, 162). Both of these cytokines have been demonstrated to be produced at the sites of active fibrosis by activated inflammatory cells, including alveolar macrophages (42, 109, 129, 158, 163). A study of the individual cytokines *in vitro* has revealed that the cytokines have various roles in the regulation of the fibrotic process (42, 109, 129). Of the many cytokines, it appears that TGF-β is probably the most important cytokine in terms of the direct stimulation of lung matrix expression which typifies fibrosis (42, 160, 162). However, accumulating evidence indicates that the fibrosis process is more complex than any one cytokine can explain, and suggests that other cytokines, such as TNF-α may orchestrate important roles in pulmonary diseases, such as idiopathic pulmonary fibrosis, sarcoidosis and tuberculosis (42, 160, 162).

The study of tumor necrosis factor-α production by alveolar macrophages is a relatively new area of research (58, 150, 164). Tumor necrosis factor-α is involved in many lung diseases including pulmonary sarcoidosis and tuberculosis (164). Past studies have illustrated the ability of bronchoalveolar lavage macrophages to produce TNF-α only in response to stimulation with the *E. coli* endotoxin lipopolysaccharide (57, 164). This indicates that TNF-α is produced by stimulated alveolar macrophages, but not by naive macrophages (164). Further research examining the production of TNF-α by patients with sarcoidosis and tuberculosis has indicated that pulmonary macrophages of these patients release significantly more TNF-α than those not infected with these illnesses (150, 164).

Chronic lung disease (CLD) is a common respiratory disorder of preterm infants (58, 155, 163, 165). The lungs of these infants have markedly increased fibroblast proliferation as well as collagen and fibronectin levels (155, 163, 165). Examinations of the bronchoalveolar fluid from these infants suggest that IL-1, IL-6, and IL-8 are present in higher levels than in children who do not develop this disease;

although it is believed that the initial fibrosis of the diseased lungs in these patients is mediated by transforming growth factor-β and tumor necrosis factor-α (58, 155, 163, 165). This finding is related to the discovery that both active and latent levels of TGF-β as well as total levels of TNF-α are increased in the pulmonary lavage fluid of infants who developed CLD (155, 165). At present, although there remains much speculation as to the reasons for the presence of certain cytokines in the diseased lungs of CLD infants, there are no firm hypotheses as to why some cytokines are present and not others. Furthermore, no hypotheses have been presented as to what, if anything is triggering the release of these cytokines from alveolar immune tissues, including the alveolar macrophage (155, 165).

Although much research has studied the involvement of cytokines present in the many diseases, including CLD, IPF and tuberculosis, very little effort has been spent on resolving the effects of various substances, like norepinephrine, on the levels or the presence of these cytokines (109, 155, 166, 167). Links between the nervous system and immune system have been established in previous research, and throughout this research project, however, these connections have not been examined in regards to the effect of catecholamines on the production of TNF-α and TGF-β by alveolar macrophages. It is important to determine whether these neuroimmunological interconnections are present in the respiratory system.

The final objective of this research project, and topic for this chapter is the modulation of TNF-α and TGF-β production by LPS stimulated and non-stimulated murine alveolar macrophages in response to the catecholamine, norepinephrine.

MATERIALS AND METHODS

MATERIALS

A complete listing of the source and all types of media, solutions, specific chemicals and equipment used in this thesis, outlined in the methods section is attached as Appendix A.

METHODS

Animals. Male, CR1:CD-1 (ICR)BR specific pathogen-free mice were used between six weeks and six months of age. Mice were housed in groups of five on woodchip bedding and were provided autoclaved food and water ad libitum. The requirements of the University's Animal Care and Ethics Committee were met for all experimental procedures

Murine bronchoalveolar lavage and murine alveolar macrophage collections. Each mouse was individually euthanized with carbon dioxide and bronchoalveolar lavage fluid was collected as per previously described methods to be analyzed for either TNF-α or TGF-β concentrations after activation with LPS and a catecholamine as described below (151, 168). Alveolar macrophages were obtained by cannulating the murine trachea, instilling and retrieving syringed 1 mL aliquots of sterile, warmed RPMI-1640 supplemented with 5% heat inactivated FBS. The entire lavage volume was centrifuged at 1000 RPM for 10 minutes at 4°C, and the cell pellet resuspended in RPMI-1640 supplemented with 10% FBS, 1.0×10^{-3} M sodium pyruvate, 2.0×10^{-3} M L-glutamine, and 5 mg/mL gentamycin sulfate.

The cell count was adjusted to 1.0×10^{6} cells/mL and aliqouted at 100 μL/well of a 96 well tissue culture treated microtitre plate. Therefore the final cell

concentration of each well was 1.0×10^5 cells/well. Cell viability, determined by exclusion of trypan blue, was > 95% in all experiments.

In vitro effects of catecholamines on stimulated and non-stimulated murine alveolar macrophages. Stimulated and non stimulated alveolar macrophages obtained from bronchoalveolar lavage, as previously described, were cultured in the presence of norepinephrine, terbutaline, a (1:1) norepinephrine-phentolamine solution, or a (1:1) norepinephrine-propranolol solution at a concentration of 10^{-6} M for 24 hrs and 48 hrs. At each of the time course points, the conditioned media was collected as described below. The alveolar macrophages which were stimulated were treated with 15.6 μg/mL lipopolysaccharide (LPS) (*Escherichia coli* 0111:84; Sigma Chemical Co.) at the time of catecholamine addition (84, 169).

After aliquoting the 1.0×10^5 cells/well, the macrophages were allowed to adhere for a minimum of 2 hours. The plates were then washed twice with RPMI-1640 supplemented with 5% FBS and cultured in 100 μL RPMI-1640 with 10% FBS, 1.0×10^{-3} M sodium pyruvate, 2.0×10^{-3} M L-glutamine, 5 mg/mL gentamycin sulfate, with or without the LPS stimulant and one of the concentrations of the adrenergic drugs and incubated for a two day time course at 37°C, 95% CO_2. Over the two day time course, at 24 hr intervals, samples of conditioned media were collected for cytokine sampling. Those samples which were tested for TGF-β were collected in the presence of 1 μg/mL pepstatin, 0.5 μg/mL aprotinin, and 0.5 μg/mL leupeptin in siliconized, sterile microfuge tubes and frozen at -80°C until ready for TGF-β quantification and isoform characterization. Samples quantified for TNF-α content were collected in sterile microfuge tubes and frozen at -80° C until ready for TNF-α quantification. A minimum of three mice were sacrificed for study of TGF-β production and a minimum of three mice were sacrificed for study of TNF-α production.

TGF-β assay. The TGF-β activity of the collected conditioned media was determined by the *in vitro* A375 assay as described in Chapter 3, however, prior to the use of the A375 cell assay to quantify TGF-β samples, the assay was calibrated with various concentrations of LPS, to determine whether LPS would have any effect on the sensitivity of the assay (71). Four concentrations of LPS were selected and prepared in the media in which the standards of rTGF-β were prepared. It is important that the LPS concentration be great enough to activate the alveolar macrophages, however, it must not have any effect on the sensitivity of the TGF-β assay or the A375 cells themselves.

TGF-β Statistical analysis. The data are expressed as total TGF-β content as well as % active and % latent of total TGF-β content. Values were compared using the Minitab Statistical Software for ANOVA. All values are reported as the mean ± the standard deviation and analyzed by Tukey's pairwise comparison test (n=6). An alpha error of 0.05 was used to test for statistical significance.

TNF-α assay. The TNF-α activity of the collected conditioned media was determined by the *in vitro* WEHI-13VAR assay as described in Chapter 2, however, prior to the use of the WEHI-13VAR cell assay to quantify TNF-α samples, the assay was calibrated with various concentrations of LPS, to determine whether LPS would have any effect on the sensitivity of the assay. Four concentrations of LPS were selected and prepared in the media in which the standards of rTNF-α would be prepared. It is, again, important that the LPS concentration be great enough to activate the alveolar macrophages, however, it must not have any effect on the sensitivity of the TNF-α assay or the WEHI-13VAR cells themselves (93).

TNF-α Statistical analysis. The data are expressed as total TNF-α content. Values are compared using the Minitab Statistical Software for ANOVA. All values are reported as the mean ± the standard deviation and analyzed by Tukey's pairwise comparison test (n=6). An alpha error of 0.05 was used to test for statistical significance.

RESULTS

Modification and Calibration of the WEHI-13VAR Tumor Necrosis Factor-α and A375 Transforming Growth Factor-β MTT Bioassays

Effects of Lipopolysaccharide on TNF-α and TGF-β Sensitive assays. To allow for determination of the effect of lipopolysaccharide on the WEHI-13VAR and A375 assays for measurement of TNF-α and TGF-β, respectively, the assays were tested with two concentrations of LPS in conjunction with the previously defined standard curve (Chapter 2 for TNF-α and Chapter 3 for TGF-β). Doses of LPS equal to, as well as above and below, that suggested as best stimulator of macrophage activation were sampled with the TNF-α and TGF-β standard and were observed to have no significant effect on the ability of the assays to quantify the standard utilizing recombinant cytokines (Figure 1 and Figure 2). All levels of LPS provided measurements of their respective cytokines similar to the control media which contained no LPS. In literature to date, the recommended dosage of LPS to promote stimulation of macrophages is 15.6 μg/mL (94). This was therefore selected as the stimulation dose for all subsequent experiments involving LPS stimulation.

Adrenergic Agonist and Antagonist Treatments, LPS Stimulated and Non-Stimulated Murine Alveolar Macrophages and Tumor Necrosis Factor-α

A measurement of the effects of various adrenergic agonist and antagonist treatments on the production of TNF-α were made over a two day time course. The

FIGURE 1. WEHI-13VAR cells (1.5×10^5 cells/mL) were utilized to test whether LPS had an effect on the rTNF-α standard curve previously established (Chapter 2 - Figure 1). Three concentrations of LPS (31.2 ug/mL, 15.6 ug/mL and 7.8 ug/mL) and a control of 0 ug/mL LPS were added to the media in which the standards of rTNF-α were to be prepared. Subsequently dilutions of rTNF-α ranging from 250 ng/mL to 0 ng/mL were prepared in these medias and added at a volume of 50uL/well. To each well, the solution of 0.02 ug/mL actinomycin was also added (50 uL/well) and after incubation the plate was read. The higher concentrations of TNF-α produced low optical density readings, while the low concentrations of TNF-α produced high optical density readings. The control media prepared without LPS showed no difference from the medias which contained one of the three concentrations of LPS. Each point on the graph represents the mean of a triplicate, with the standard deviation of the mean represented by error bars.

Legend:
- ● - 0 ug/mL LPS
- ▲ - 31.2 ug/mL LPS
- ▼ - 15.6 ug/mL LPS
- ■ - 7.8 ug/mL LPS

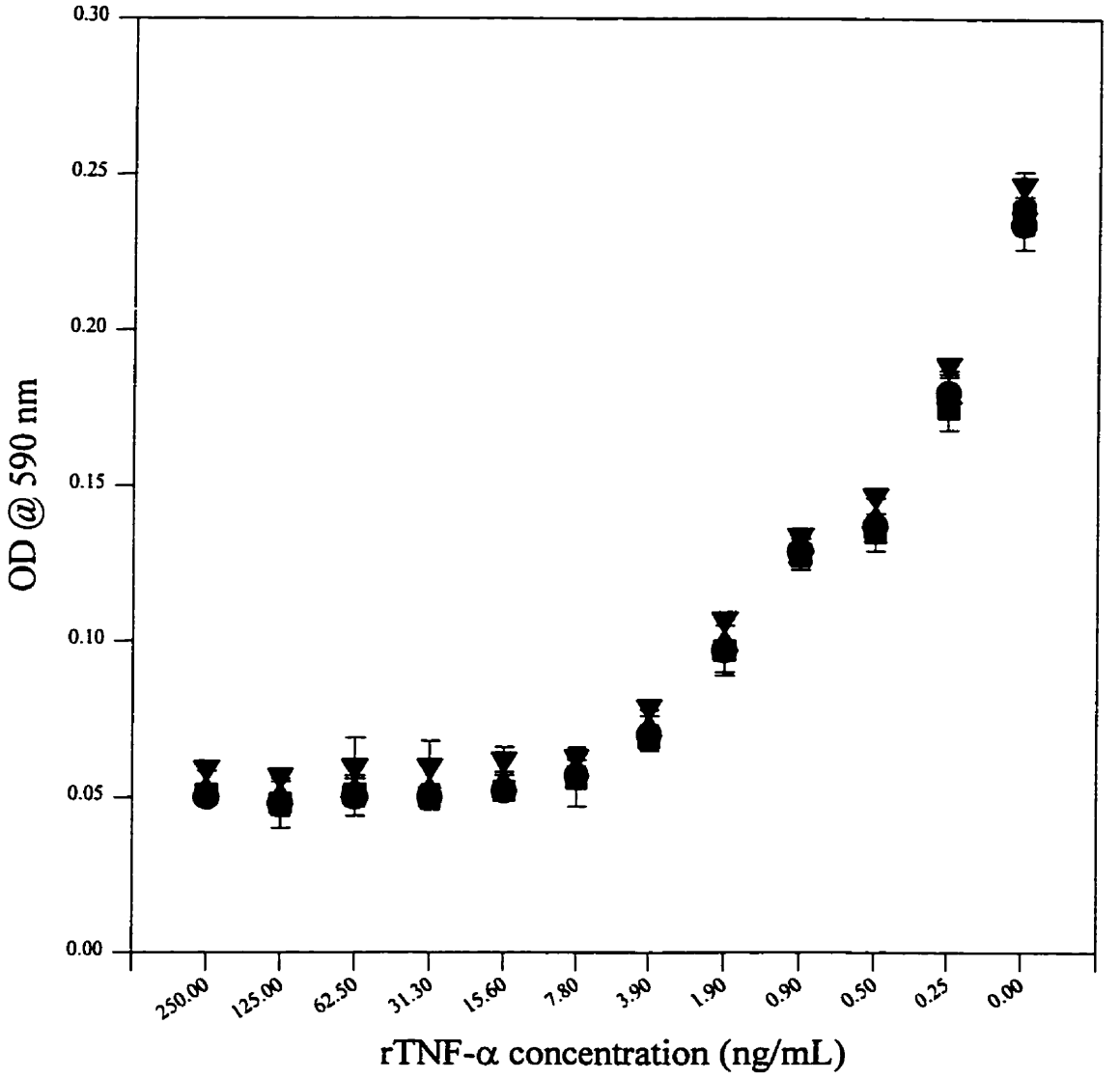
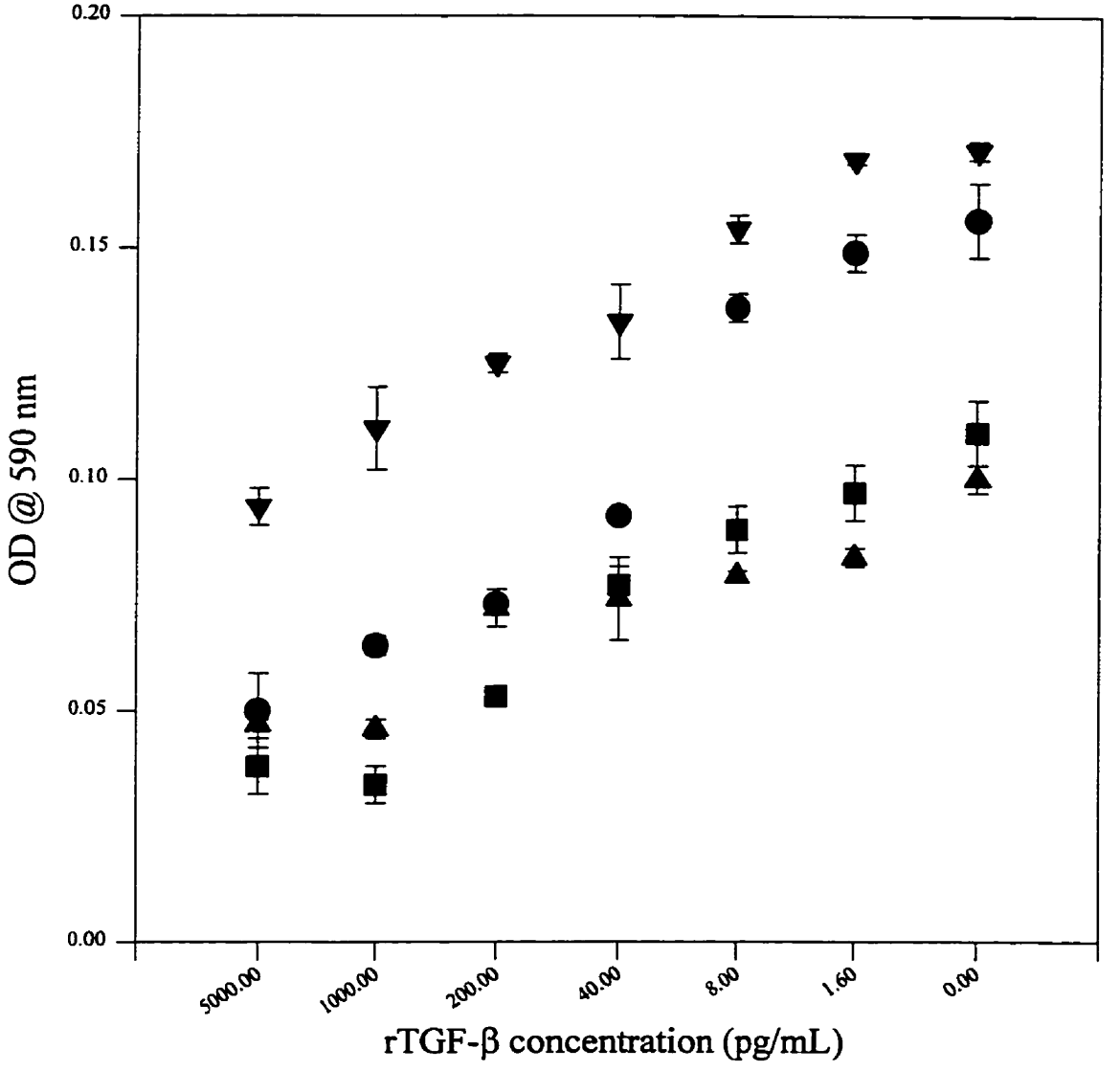

0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD @ 590 nm
250.00
125.00
62.50
31.30
15.60
7.80
3.90
1.90
0.90
0.50
0.25
0.00
rTNF-α concentration (ng/mL)

FIGURE 2. A375 cells (1.5×10^4 cells/mL) were utilized to test whether LPS had an effect on the rTGF-β standard curve previously established (Chapter 3 - Figure 1). Three concentrations of LPS (31.2 ug/mL, 15.6 ug/mL and 7.8 ug/mL) and a control of 0 ug/mL LPS were added to the media in which the standards of TGF-β were to be prepared. Subsequently dilutions of TGF-β ranging from 5000 pg/mL to 0 pg/mL were prepared in these medias and added at a volume of 100 uL/well. The higher concentrations of TGF-β produced low optical density readings, while the low concentrations of TGF-β produced high optical density readings. The control media prepared without LPS showed no difference from the medias which contained one of the three concentrations of LPS. Each point on the graph represents the mean of a triplicate, with the standard deviation of the mean represented by error bars.

Legend:
- ● - 0 ug/mL LPS
- ▲ - 31.2 ug/mL LPS
- ▼ - 15.6 ug/mL LPS
- ■ - 7.8 ug/mL LPS


0.20
0.15
0.10
0.05
0.00
OD @ 590 nm
5000.00
1000.00
200.00
40.00
8.00
1.60
0.00
rTGF-β concentration (pg/mL)

adrenergic treatments consisting of norepinephrine, terbutaline, a 1:1 norepinephrine-phentolamine solution and a 1:1 norepinephrine-propranolol solution were added at a concentration of 10^{-6} M and were utilized to determine whether α-agonists and/or β-agonists could modulate the production of TNF-α by non-stimulated murine alveolar macrophages over a two day time course. The results of these experiments are shown in Figure 3. Subsequently, the addition of LPS (15.6 ug/mL) to the media in which the adrenergic agonists and antagonists were prepared was measured to determine the effects of the adrenergic agonists and antagonists on TNF-α production by LPS-stimulated alveolar macrophages. These results are also illustrated in Figure 3.

From the results, it appears that norepinephrine significantly decreases the production of TNF-a by alveolar macrophages stimulated with LPS. This effect may be modulated through the β-adrenoceptor of the alveolar macrophage since NE when combined with the α-adrenergic antagonist PH (NPH), demonstrates an increases in the inhibitory effect, so that overall TNF-α production is negligible. Because PH is an α-adrenergic antagonist, it should block any effect of NE modulation through the α-adrenoceptor. Therefore it appears that when the treatment of NPH was applied in combination with LPS, the β-adrenoceptor of the murine alveolar macrophage was still accessible by NE and thus NE was able to inhibit production of TNF-α by the murine alveolar macrophage.

A β_2-adrenergic agonist, terbutaline, was sampled with the alveolar macrophages, however, it appears that this β_2-adrenoceptor agonist at a concentration of 10^{-6} M had no significant effect on the production of TNF-α by LPS stimulated and non-stimulated alveolar macrophages. Likely, either the concentration of TE was ineffective, or perhaps the β-adrenoceptor through which NE is modulating its effect is the β_1-adrenoceptor, not the β_2-adrenoceptor. Although TE has affinity for both the β_1- and the β_2-adrenoceptors, it possesses efficacy for the β_2-adrenoceptor (50).

FIGURE 3. A comparison of TNF-α production over a two day time course by LPS stimulated and non-stimulated murine alveolar macrophages treated with various adrenergic agonists and antagonists. The results illustrate the mean of the total TNF-α production (n=3) for the experiment at 24 hrs (shaded bars) and 48 hrs (hatched bars). The standard deviation of the mean is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

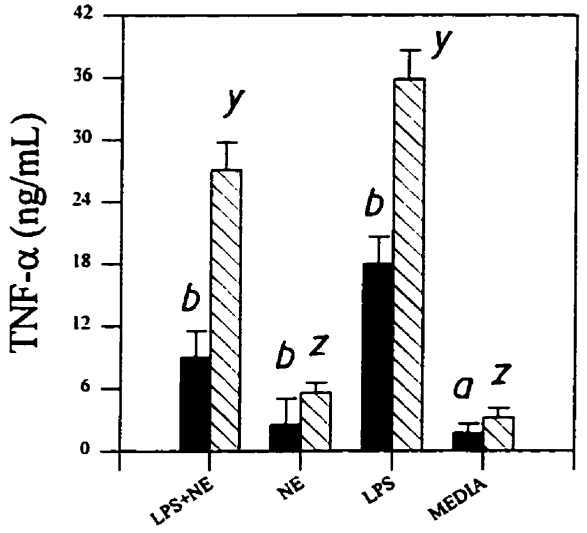
Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	NPR	≤0.001	≤0.001
TE	≤0.001	≤0.001	NPH	≤0.001	≤0.001

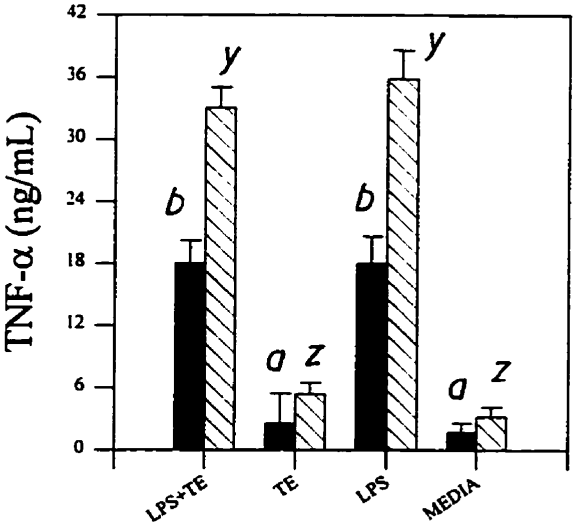
Adrenergic Treatment Abbreviations:

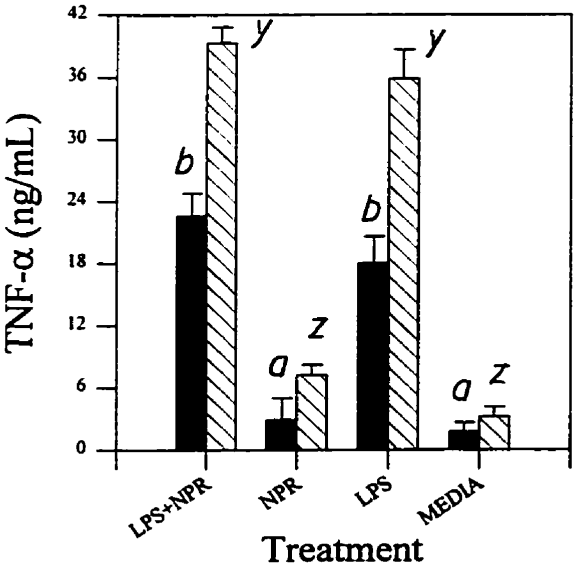
NE: Norepinephrine
TE: Terbutaline
NPR: Norepinephrine + Propranolol
NPH: Norepinephrine + Phentolamine

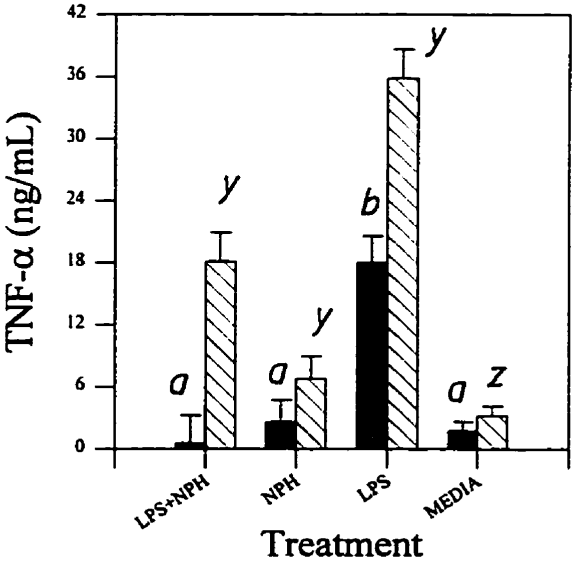
Adrenergic Treatment Concentrations:

(-6): 10(-6) M


TNF-α (ng/mL)
42
36
30
24
18
12
6
0
b
y
b
z
b
y
a
z
LPS+NE
NE
LPS
MEDIA


TNF-α (ng/mL)
42
36
30
24
18
12
6
0
b
y
a
z
b
y
a
z
LPS+TE
TE
LPS
MEDIA


TNF-α (ng/mL)
42
36
30
24
18
12
6
0
b
y
a
z
b
y
a
z
LPS+NPR
NPR
LPS
MEDIA
Treatment


TNF-α (ng/mL)
42
36
30
24
18
12
6
0
a
y
a
y
b
y
a
z
LPS+NPH
NPH
LPS
MEDIA
Treatment

The experimental results illustrate that the treatment of LPS + NPR had a stimulatory effect on the production of TNF-α by the alveolar macrophages, and this effect appears to be significant. PR is an β-adrenoceptor antagonist, and therefore should block the ability of norepinephrine to bind to the β-adrenoceptor of the macrophage. Therefore, it appears that NE, when in combination with PR, can and will bind to the α-adrenoceptor of the macrophage. Since norepinephrine is noted to act through both the α- and β-adrenoceptors of the macrophage, these observations are possible, and further serve to illustrate the results obtained in these experiments. It appears that norepinephrine has a preference for the β-adrenoceptor of the alveolar macrophage, and when in combination with the β-adrenoceptor will cause an inhibitory effect on the production of TNF-α. When NE is able only to access the macrophage through the α-adrenoceptor, it appears to have a stimulatory effect on TNF-α production. The adrenergic treatments applied without LPS to the murine alveolar macrophages had no significant effect on the production of TNF-α as compared to the media control.

Evaluating the above data for total TNF-α production by alveolar macrophages over a two day time period using Tukey's pairwise comparison illustrates that there are some reliable differences between some adrenergic treatments, but not others (Figure 3). Finally, the addition of anti-TNF-α antibodies to duplicate samples of conditioned media demonstrated that the effects illustrated in Figure 3 were in part due to TNF-α, with the TNF-α present in the conditioned media being neutralized by the antibody confirming the specificity of the WEHI-13VAR bioassay in detecting TNF-α.

Adrenergic Agonist and Antagonist Treatments, LPS Stimulated and Non-Stimulated Murine Alveolar Macrophages and Transforming Growth Factor-β

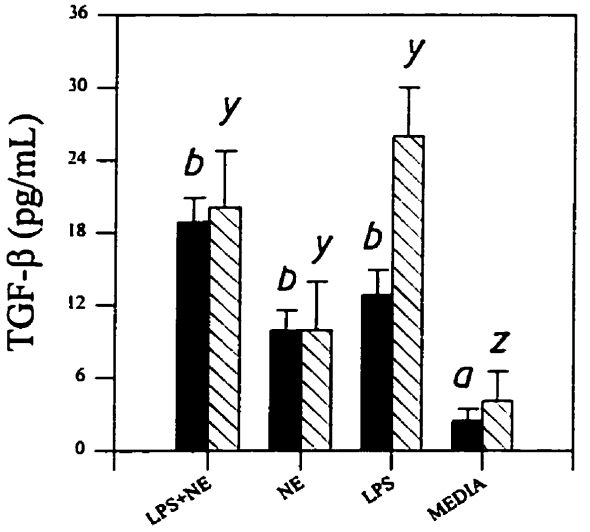
A measurement of the effects of various adrenergic agonist and antagonist treatments at a single concentration over a two day time period was completed to determine their effect on TGF-β production by murine alveolar macrophages. This was again done by utilizing either norepinephrine, terbutaline, a 1:1 norepinephrine-phentolamine solution or a 1:1 norepinephrine-propranolol solution. The drugs were added to the alveolar macrophages at a concentration of 10^{-6} M and measurements were completed to determine whether α-agonists and/or β-agonists could modulate the production of total TGF-β by murine alveolar macrophages The results of these experiments over the time course are shown in Figure 4. Subsequently, the addition of LPS (15.6 ug/mL) to the media in which the adrenergic agonists and antagonists were prepared was measured to determine the effects of the adrenergic agonists and antagonists on total TGF-β production by LPS-stimulated alveolar macrophages.

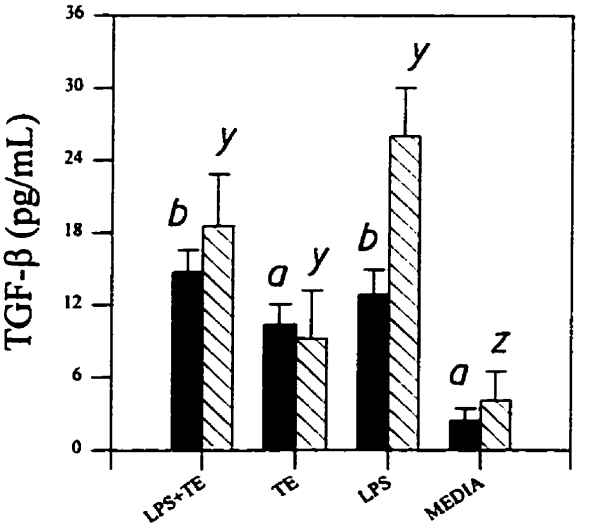
It is illustrated at 24 hrs, NE, TE and the 1:1 NPR treatment all served to increase total TGF-β production and the effect of NE is significant. It appears that the stimulatory effect of the adrenergic treatment might possibly be modulated through the β-adrenoceptor since both NE and TE when individually combined with LPS had similar effects on increasing total TGF-β production.

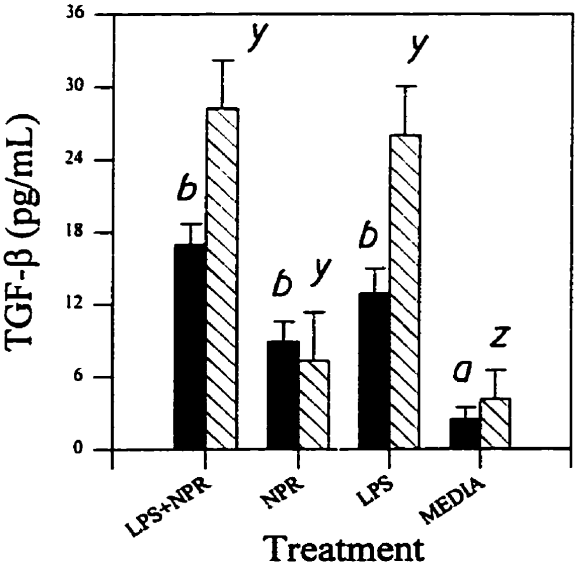
At 48 hrs, the results display that NE significantly decreases the production of total TGF-β by murine alveolar macrophages stimulated with LPS. This inhibitory effect again appears to be modulated through the β-adrenoceptor as treatments of the stimulated alveolar macrophages with TE produced an even further inhibitory effect on total TGF-β production. Also, when NE was combined with PH (NPH) in a 1:1 ratio, the inhibitory effects are again significant, with the decreases in total TGF-β production levels of this treatment similar to those observed with the TE treatment. Because PH is an α-adrenergic antagonist, it should block any effect of NE

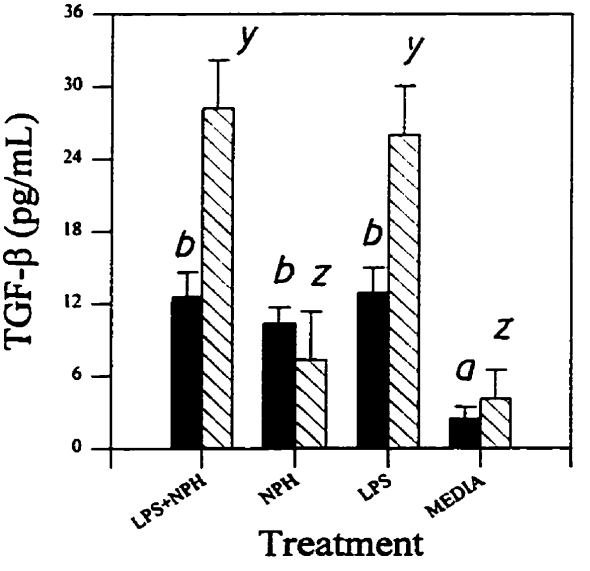
FIGURE 4. A comparison of total TGF-β production over a two day time course by LPS stimulated and non-stimulated murine alveolar macrophages treated with various adrenergic agonists and antagonists. The results illustrate the mean of the total TGF-β production (n=3) for the experiment at 24 hrs (shaded bars) and 48 hrs (hatched bars). The standard deviation of the mean is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.001	NPR	≤0.001	≤0.001
TE	≤0.001	≤0.001	NPH	≤0.001	≤0.001


TGF-β (pg/mL)
36
30
24
18
12
6
0
b
y
b
y
b
y
a
z
LPS+NE
NE
LPS
MEDIA


TGF-β (pg/mL)
36
30
24
18
12
6
0
b
y
a
y
b
y
a
z
LPS+TE
TE
LPS
MEDIA


TGF-β (pg/mL)
36
30
24
18
12
6
0
b
y
b
y
b
y
a
z
LPS+NPR
NPR
LPS
MEDIA
Treatment


TGF-β (pg/mL)
36
30
24
18
12
6
0
b
y
b
z
b
y
a
z
LPS+NPH
NPH
LPS
MEDIA
Treatment

modulation through the α-adrenoceptor, therefore it appears that when the treatment of NPH was added to the LPS stimulated population of alveolar macrophages the NE was still able to inhibit total TGF-β production through the β-adrenoceptor.

The β_2-adrenergic agonist terbutaline, as mentioned, inhibited the production of total TGF-β production by stimulated alveolar macrophages as compared to the LPS stimulated population. Further studies are likely necessary to characterize the adrenoceptor subtype responsible for mediating this inhibitory response because although TE is recognized for its efficacy for the β_2-adrenoceptor, it does have affinity for both the β_1- and β_2-adrenoceptors (50).

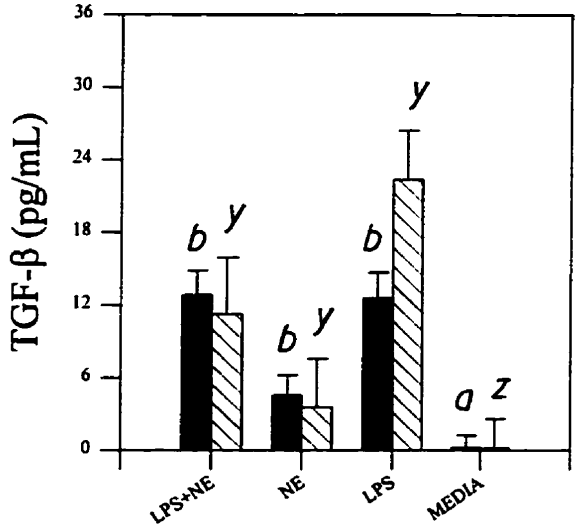
Examining the outcomes of the NPR treatment illustrates that NPR was able to augment significantly the production of total TGF-β by LPS stimulated and non-stimulated murine alveolar macrophages. Since PR is a β-adrenergic antagonist, it should eliminate the β-adrenoceptor effect of NE. This appears to have occurred, illustrating that exposure of NE to only the α-adrenoceptor increases the production of total TGF-β by LPS stimulated murine alveolar macrophages as compared to the LPS control population.

The adrenergic agonist and antagonist treatments appear to have had significant effects on the production of active TGF-β by the murine alveolar macrophage samples over the two time course points (Figure 5). The peak production of active TGF-β by the media control sample of non-stimulated murine splenocytes occurred at 48 hrs.

Evaluating the data for total TGF-β production by alveolar macrophages over a two day time period using Tukey's pairwise comparison illustrates that there are some significant differences between some adrenergic treatments, but not others (Figure 5 and 6). Finally, the addition of anti-TGF-β_1 antibodies to duplicate samples of conditioned media demonstrated that the effects illustrated in Figure 4 were due to

FIGURE 5. A comparison of active TGF-β production over a day time course by LPS stimulated and non-stimulated murine alveolar macrophages treated with various adrenergic agonists and antagonists. The results illustrate the mean of the active TGF-β production (n=3) for the experiment at 24 hrs (shaded bars) and 48 hrs (hatched bars). The standard deviation of the mean is represented by error bars. Treatment bars which do not have the same letter as their respective time course media control treatment are noted to be statistically different utilizing Tukey's Pairwise Comparison. The determined ANOVA p-value for the 24 hr and 48 hr results is displayed below, arranged according to adrenergic treatment.

Adrenergic Treatment	ANOVA p-value 24 hr	48hr	Adrenergic Treatment	ANOVA p-value 24 hr	48 hr
NE	≤0.001	≤0.002	NPR	≤0.001	≤0.001
TE	≤0.001	≤0.001	NPH	≤0.001	≤0.001


TGF-β (pg/mL)
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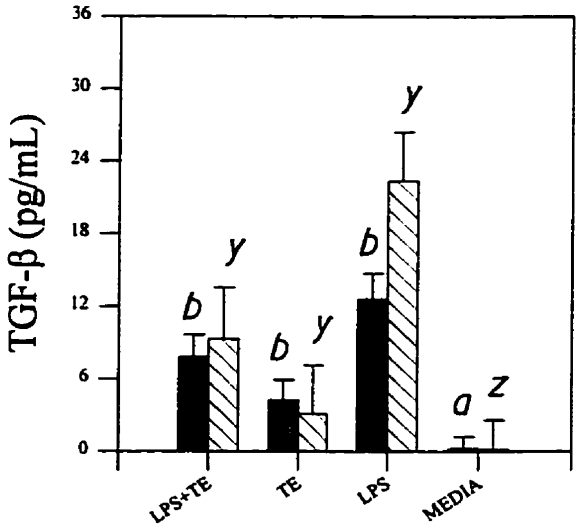
30
24
18
12
6
0
b
y
b
y
b
y
a
z
LPS+NE
NE
LPS
MEDIA


TGF-β (pg/mL)
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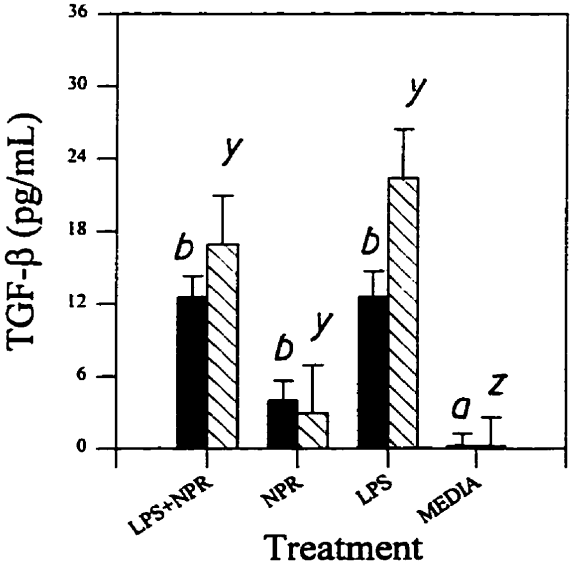
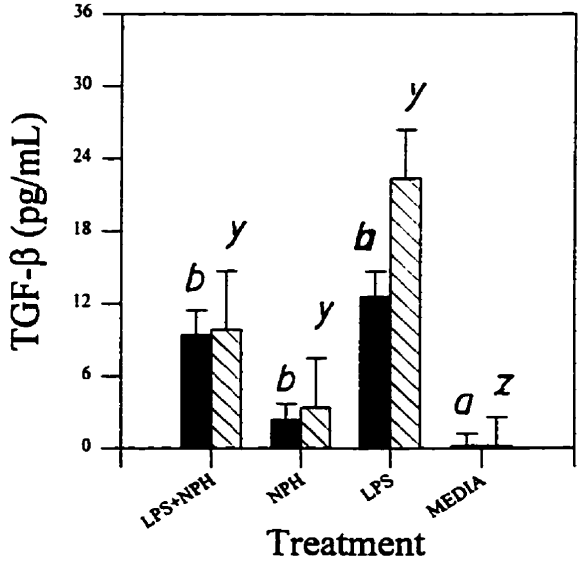
30
24
18
12
6
0
b
y
b
y
b
y
a
z
LPS+TE
TE
LPS
MEDIA


TGF-β (pg/mL)
36
30
24
18
12
6
0
b
y
b
y
b
y
a
z
LPS+NPR
NPR
LPS
MEDIA
Treatment


TGF-β (pg/mL)
36
30
24
18
12
6
0
b
y
b
y
b
y
a
z
LPS+NPH
NPH
LPS
MEDIA
Treatment

TGF-β_1, with almost all TGF-β present in the conditioned media being neutralized by the antibody (119, 128, 133, 143, 151, 170).

Effects of adrenergic agonist and antagonist treatments on the production of biologically latent and active TGF-β by LPS-stimulated and non-stimulated alveolar macrophages. TGF-β was quantified both before and after activation by acidification because TGF-β obtained from most cells, including platelets and tissues is normally not in a biologically active form. The results of the acidification process of the total TGF-β obtained in the experiment (Figure 4) are displayed in Figures 6 as % latent (shaded bars) versus % active (black bars) TGF-β.

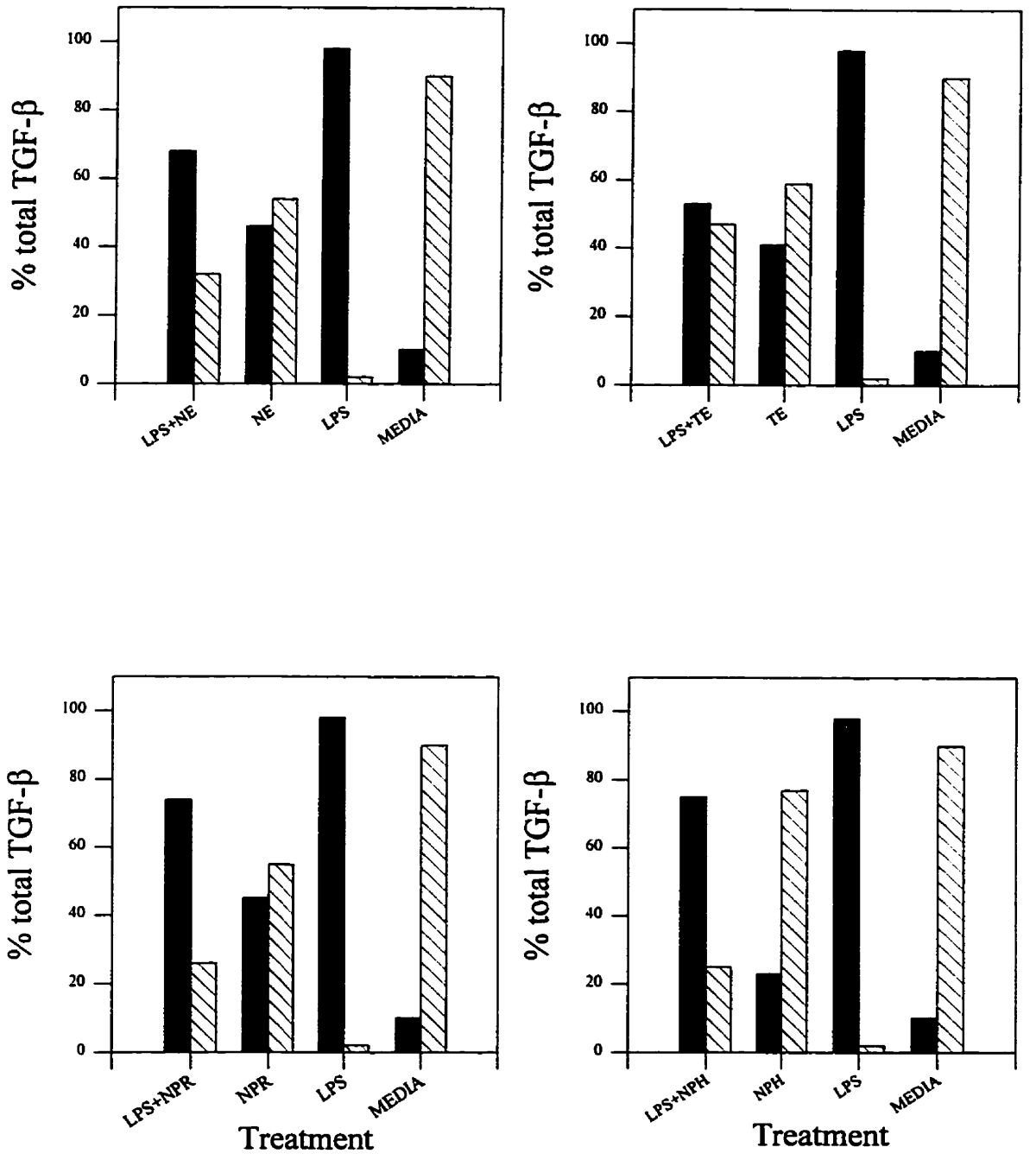
It is visible from the figures that alveolar macrophages activated with LPS or LPS + an adrenergic drug had increased levels of active TGF-β, while alveolar macrophages stimulated solely with an adrenergic drug or media had higher levels of latent TGF-β. This pattern is observed over the two day time course and suggests that activated cells produce larger quantities of active TGF-β than those cells which were not-previously stimulated with LPS.

DISCUSSION

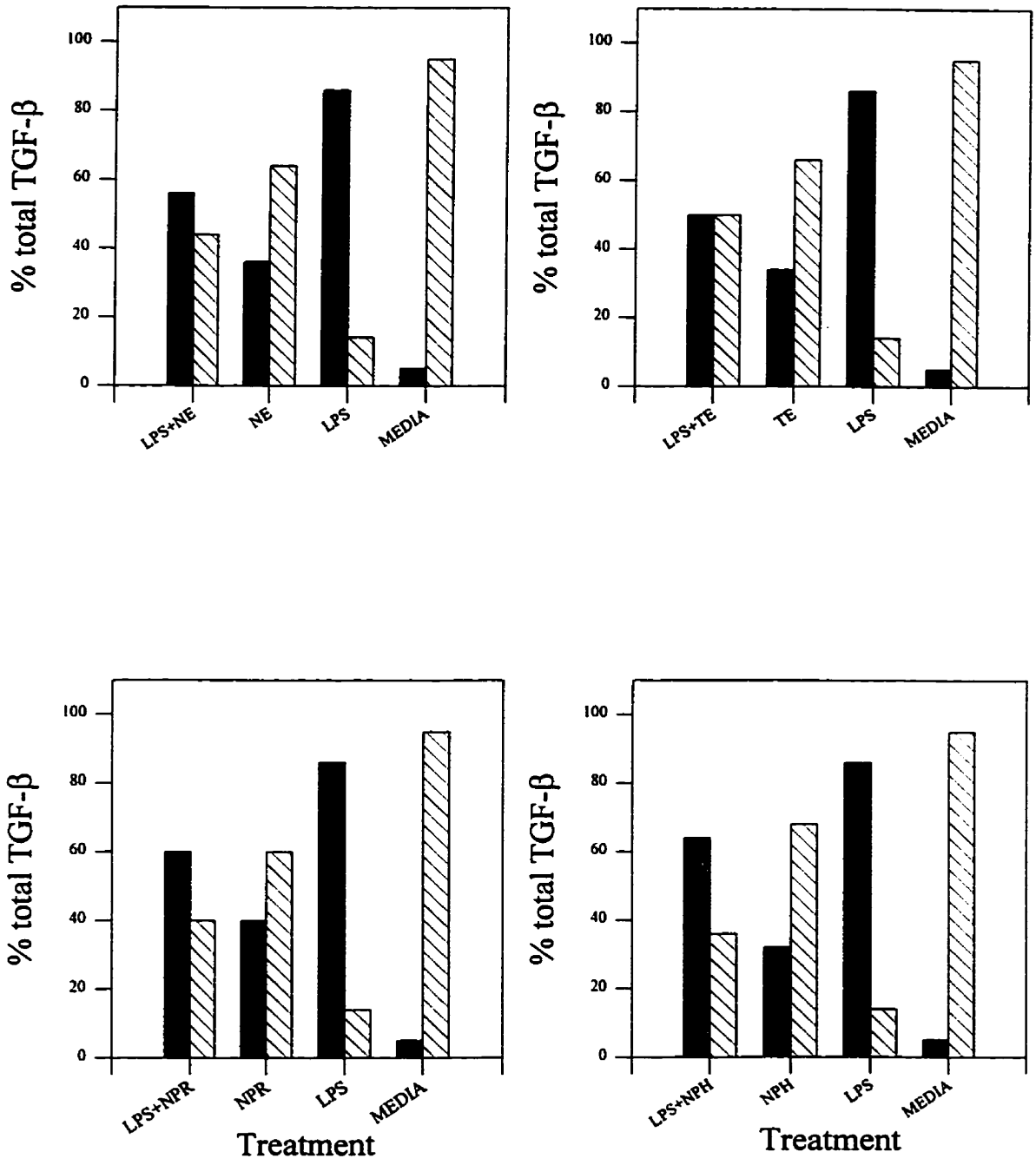
Transforming growth factor-β and tumor necrosis factor-α are two important cytokines in disease (48, 159, 171). TNF-α, a member of the proimflammatory cytokines, is able to mediate many of the powerful inflammatory responses mounted by the body in the face of several different illnesses while TGF-β is important in tumour development, extracellular matrix protein synthesis and immunosuppression (48, 85). Because both of these cytokines play important roles in numerous diseases, the necessity of determining their mechanisms of *in vivo* regulation is of fundamental clinical importance (48, 85).

FIGURE 6. The biological activity of total TGF-β produced by LPS stimulated and non-stimulated murine alveolar macrophages treated with various adrenergic agonists and antagonists. Based on the total TGF-β concentrations obtained through experimentation (Figure 4), calculations of % latent TGF-β (hatched bars) compared to % active TGF-β (shaded bars) were completed. Figure 5A demonstrates the mean activity of the TGF-β in the samples collected at the 24 hour time point and Figure 5B -- the mean activity at 48 hrs.

A


% total TGF-β
100
80
60
40
20
0
LPS+NE
NE
LPS
MEDIA
% total TGF-β
LPS+TE
TE
LPS
MEDIA
% total TGF-β
LPS+NPR
NPR
LPS
MEDIA
Treatment
% total TGF-β
LPS+NPH
NPH
LPS
MEDIA
Treatment

B


% total TGF-β
100
80
60
40
20
0
LPS+NE
NE
LPS
MEDIA
LPS+TE
TE
LPS
MEDIA
LPS+NPR
NPR
LPS
MEDIA
Treatment
LPS+NPH
NPH
LPS
MEDIA
Treatment

The results of this research project illustrate that through the sympathetic neurotransmitter norepinephrine, the production of both TGF-β and TNF-α by LPS-induced alveolar macrophages is inhibited through β-adrenoceptor modulation. These results are significant in both the manner which norepinephrine modulates the production of the two cytokines as well as their effects and potential effects on disease progression.

The Modulation of Tumor Necrosis Factor-α Production by Lipopolysaccharide Stimulated and Non-Stimulated Murine Alveolar Macrophages with Adrenergic Agonist and Antagonist Treatments

The results from this study illustrate that norepinephrine is able to significantly decrease the production of TNF-α by LPS-stimulated alveolar macrophages. Additionally, when added in a 1:1 ratio with phentolamine, norepinephrine produced an even further decrease in TNF-α production suggesting involvement of the β-adrenoceptor of the murine alveolar macrophage. Subsequently when norepinephrine was added in a 1:1 ratio with propranolol, a significant increase in TNF-α production was observed -- again suggesting the possibility of a β-adrenoceptor effect, and also potentially the involvement of the α-adrenoceptor. These findings are supported by numerous research papers focusing on murine splenic populations (172, 173, 174, 175).

A number of studies have demonstrated that activation of murine splenic macrophage β-adrenergic receptors results in the suppression of TNF-α production (172, 173, 175). In fact, previous data has shown that stimulation of the β-adrenoceptor inhibits the production of TNF-α in response to LPS stimulation (169, 172, 173, 175). Even though research has been published regarding the stimulation of the β-adrenoceptor on macrophages to inhibit the production of TNF-α, research has

yet to characterize the β-adrenoceptor subclass to which norepinephrine is binding and thus provoking a response.

In this experiment, the failure of terbutaline, a β_2-agonist, to inhibit TNF-α production by stimulated alveolar macrophages can thus be interpreted to mean that norepinephrine is having an inhibitory effect through the β_1-adrenoceptor of the alveolar macrophage. Terbutaline's lack of inhibition on TNF-α production can also be explained either by the possible down-regulation of β-adrenoceptors by macrophages that is known to occur in the presence of β-agonists, or may be due to the inappropriate concentration of the drug in the experiments (169, 174, 176). Thus, in order to better determine the adrenoceptor through which NE is modulating it's effects, more experiments with other β-agonists and antagonists as well as other α-agonists and antagonists are necessary, and these future experiments must be performed at various concentrations of the adrenergic drugs.

Although previous studies, as well as this study, have demonstrated that β-adrenergic stimulation causes the inhibition of TNF-α production by splenic and alveolar macrophages, research has indicated that catecholamines can have a dual effect on LPS-induced TNF-α production, depending on whether α- or β-adrenoceptors are stimulated
(169, 174, 177). Many scientists including Splenger *et al.* have found that stimulation of the α_2-adrenoceptor increases LPS induced macrophage TNF-α production (169, 175, 177). This result illustrates the ability of norepinephrine to bind to opposing receptors depending on the concentration of the neuroendocrine hormone and also the concentrations of the receptor population on the macrophage (169, 175). In the findings of many other laboratories, data have illustrated that norepinephrine modulates the production of TNF-α in a dose dependent manner (59, 85, 175, 177). In fact, high doses of norepinephrine, greater than 10^{-7} M have been noted to inhibit the production of TNF-α through the β-adrenoceptor, while low doses (less than 10^{-8}

M) have been shown to bind to the α-adrenoceptor and subsequently increase the production of TNF-α by activated macrophages (59, 84, 85). Theoretically, this increase in TNF-α production by stimulation of the α_2-adrenoceptor site is most likely due to activation of protein kinase C, while the inhibition of TNF-α production is likely due to the well-documented ability of β-agonists to stimulate adenylate cyclase activity and thus increase cAMP synthesis (59, 84, 169, 175). In this Masters research project, a dose of norepinephrine equal to 10^{-6} M in the presence of a β-adrenergic antagonist was found to inhibit TNF-α secretion by LPS stimulated alveolar macrophages, and a dose of norepinephrine equal to 10^{-6} M in the presence of an α-adrenergic antagonist was found to increase TNF-α secretion by this same cell population.

In addressing the differing physiological effects norepinephrine has depending on whether α- or β-adrenoceptors are stimulated, one must look to many scientific papers including Spengler *et al.* (46, 59, 177) for potential solutions (53, 59, 85, 169). Splenger *et al.* (46, 59, 177) suggest that macrophage response depends not only on the receptor population of the macrophage, but also on the stores of endogenous norepinephrine within the cell (59, 85, 169, 177). Spengler discusses the potential ability of macrophages to modulate their production of TNF-α by autoregulatory mechanisms which involve endogenous norepinephrine (46, 157, 169, 177). It can be concluded through the work of Spengler that macrophages, when stimulated by LPS, release their pooled norepinephrine and autoregulate their TNF-α production (46, 169). Therefore, his work suggests that if cells have high stores of endogenous norepinephrine, their production of TNF-α is likely to be greatly inhibited when α_2-adrenergic agonists are used as stimulants, and alternatively, those macrophages with low endogenous norepinephrine levels are likely to produce greater amounts of TNF-α when α_2-adrenergic agonists are again used for stimulation. From these previous observations, endogenous catecholamines released after LPS treatment or during an

immune response via stimulation of a β-adrenoceptor could be involved *in vivo* in the inhibition of TNF-α secretion (59, 169, 174).

Evidence in this project concludes that α-antagonists inhibit TNF-α production while β-antagonists are able to increase the production of TNF-α when combined with norepinephrine. Although adrenoceptor type and concentration are major factors in TNF-α modulation by norepinephrine, it appears from this research project that norepinephrine may have a higher affinity for the β-adrenoceptor than the α-adrenoceptor. Thus, the availability of a β-adrenoceptor for NE binding results in decreased production of TNF-α by stimulated alveolar macrophages, and the ligand of norepinephrine to the α-receptor modulates the production of increased TNF-α by stimulated alveolar macrophages. This modulation of TNF-α production agrees with the suggestion that *in vivo*, the sympathetic nervous system is involved in the fine tuning of LPS induced TNF-α production through the release of catecholamines (169, 174, 175, 178).

The Modulation of Total Transforming Growth Factor-β Production by Lipopolysaccharide Stimulated and Non-Stimulated Murine Alveolar Macrophages with Adrenergic Agonist and Antagonist Treatments

Examining the effects of norepinephrine on the production of total TGF-β by alveolar macrophages is by far more confusing than the effects observed on TNF-α production. To date, no research has focused on the area of alveolar macrophage production of TGF-β modulated by norepinephrine. As with the TNF-α study, the observations regarding the production of total TGF-β by stimulated alveolar macrophages are similar -- total TGF-β was secreted in large amounts in alveolar macrophages treated with only LPS, while alveolar macrophages treated with LPS + NE secreted significantly lower amounts of total TGF-β. Again, the effect of norepinephrine on the inhibition of total TGF-β secretion was enhanced when

phentolamine was added in a 1:1 ratio with norepinephrine -- and in a 1:1 solution with propranolol, norepinephrine was able to increase the production of total TGF-β. These results suggest that norepinephrine, when acting through the α- or β-adrenoceptors of the alveolar macrophage, has an adrenoceptor specific effect on total TGF-β production. Because of these experimental results, previous studies regarding the concentration and type of adrenoceptor on the macrophage must again be examined (59, 177, 179).

The experimental data illustrate that when phentolamine was added in a 1:1 combination with norepinephrine (NPH), total TGF-β production by LPS stimulated alveolar macrophages was limited. In fact, it was more limited than when only the LPS + NE treatment was added. Examining the effects of an β-antagonist in combination with norepinephrine (NPR) shows an increased production of total TGF-β. Thus, it appears that norepinephrine, when able to bind to an α-adrenoceptor, augments the production of total TGF-β, but NE, binding to a β-adrenoceptor results in decreased total TGF-β production. When examining the results obtained when only the LPS + NE treatment was added to the alveolar macrophages, the results indicate a β-adrenoceptor effect. In this experiment, NE should be able to access either the α-adrenoceptor or β-adrenoceptor if they both are present on the macrophages. Thus, it appears that norepinephrine has a greater affinity for the β-adrenoceptor on LPS stimulated macrophages, and when it is able to bind to the β-adrenoceptor, NE will serve to modulate a decreased production in total TGF-β.

These results also illustrate why the production levels of total TGF-β, when modulated solely by NE, fall between that observed when the norepinephrine and propranolol solution (NPR) and the norepinephrine and phentolamine solution (NPH) were added to stimulated alveolar macrophages. Norepinephrine on it's own appears to have the potential to modulate a dual effect. When it binds to the macrophage through the adrenoceptors, a ligand with the α-adrenoceptor provides for increased

total TGF-β production while a ligand with the β-adrenoceptor limits total TGF-β production. Therefore, it appears that there is an overlapping in the signaling response, and the dynamic nature of the norepinephrine response depends again on the concentration, isoform, and availability of macrophage adrenoceptor.

It is also apparent that norepinephrine is able to modulate a decreased level of total TGF-β through the β-adrenoceptor, since the terbutaline treatment in this research project again displayed an overall decrease in total TGF-β production over the 72 hour time course. Since terbutaline is a β-adrenergic agonist, it is possible that norepinephrine is modulating this decrease in TGF-β production through the β-adrenoceptor. This hypothesis is also supported by the experimental data indicating that NE in combination with PH (NPH) further decreased the secreted levels of total TGF-β by the LPS stimulated murine alveolar macrophages.

The data collected in this project may also suggest that the predominant adrenoceptor type on the alveolar macrophage is the β-adrenoceptor. This hypothesis results from the result in which NE, when added to the stimulated murine alveolar macrophages produced an inhibition of both TNF-α and TGF-β quantities. However, when NE was added in combination with each of the α- and β-antagonists, adrenoceptor-specific increases and decreases in TNF-α and TGF-β production were respectively detected. From the results utilizing the β-adrenergic agonists, it appears then that either NE has an increased affinity for the β-adrenoceptor, or perhaps there is a higher probability of NE binding to these adrenoceptors due to their increased concentration on the macrophage. These preliminary hypotheses must be explored in more detail.

The Biological Activity of Transforming Growth Factor-β and Isoform Identification

TGF-β is secreted by cells in many isoforms, as well as in two biological forms -- active and latent. Because of this, it was important in this research project to not only resolve the effects of norepinephrine on the modulation of TGF-β production, but it was also imperative to determine both the isoform and biological activity of the TGF-β present in the conditioned media.

The isoform of TGF-β secreted by the LPS stimulated and non-stimulated murine alveolar macrophage was found to be TGF-β_1, because experiments with anti-transforming growth factor-β_1 antibodies present in the conditioned media at the time of TGF-β assay were effectively able to neutralize most of the TGF-β present. Based on the published research examining isoforms of TGF-β present in alveolar samples, this is, however, the expected result (128, 143, 151, 179).

There are five known isoforms of TGF-β, however, only three of these have been reported to be found in mammalian cells. Research by Khalil and Greenberg, two the of prominent researchers in the field of idiopathic pulmonary research and TGF-β supports the hypothesis that non-stimulated murine alveolar macrophages primarily secrete TGF-β_1, although small quantities of TGF-β_2 and TGF-β_3 are also present in histological studies (151). Khalil and Greenberg have additionally noted that quantities of TGF-β_1 are increased by activated murine macrophages, while TGF-β_2 and TGF-β_3 isoform amounts remain relatively unchanged. The results relating to the isoform neutralization by anti-TGF-β_1 antibodies in these research experiments suggest that the prominent TGF-β isoform present was TGF-β_1, and thus agrees with previously published literature (151).

This research project also illustrated that murine alveolar macrophages stimulated with LPS, or LPS plus an adrenergic treatment, had much higher production levels of the active form of TGF-β, while those murine alveolar

macrophages stimulated with only an adrenergic treatment produced greater quantities of latent TGF-β. These results suggest that LPS-stimulated murine alveolar macrophages produce larger amounts of active TGF-β compared to non-stimulated murine alveolar macrophages, and these results are important since only biologically active TGF-β is able to participate in immune reactions.

TGF-β is active biologically only when it has been activated by low pH, proteolysis, or by the many unknown mechanisms involved in tumor development (119, 120, 126). Therefore, it is apparent, that the secretion of latent TGF-β by cells is an important regulatory mechanism since most cells ubiquitously secrete TGF-β as well as express receptors for this cytokine. In this research project, the increased secretion of active TGF-β in response to norepinephrine is of clinical importance because in it's active form, TGF-β is known to participate in many immune responses, including the fibrosis process of Idiopathic Pulmonary Fibrosis.

Conclusions Regarding the Complex Role of Norepinephrine in the Modulation of Transforming Growth Factor-β and Tumor Necrosis Factor-α by Lipopolysaccharide Stimulated and Non-Stimulated Murine Alveolar Macrophages

In this study, norepinephrine is able to augment significantly LPS-stimulated alveolar macrophage production of both TGF-β and TNF-α over a two day time course. The results of this project support the ability of norepinephrine to modulate the production of both TGF-β and TNF-α through the β-adrenoceptor of the LPS stimulated murine alveolar macrophage.

A significant decrease in the production of total TGF-β was modulated by norepinephrine through the β-adrenoceptors of the LPS stimulated murine alveolar macrophages, while a significant increase in total TGF-β production was observed when β-adrenergic antagonists were utilized in conjunction with NE (NPR). The

biological activity of the TGF-β secreted by the alveolar macrophages suggests that LPS stimulation results in the production of active TGF-β, while a lack of LPS stimulation relates to increases in latent TGF-β production.

The production of TNF-α by LPS stimulated murine alveolar macrophages was significantly decreased in the presence of norepinephrine and also when norepinephrine was combined with phentolamine (NPH), thus suggesting that the modulation of TNF-α production is through the β-adrenoceptors of LPS stimulated murine alveolar macrophages.

This alveolar macrophage study is only in it's preliminary stages, since the number of mice utilized to collect this data was only three for each cytokine. Thus, further research is needed firstly to determine whether these effects are indeed significant in larger populations, and a much more in-depth classification of adrenoceptors and actions of norepinephrine on these receptors is also needed. This could be done utilizing specific α- and β-agonists, as well as varying their respective concentrations to determine whether the modulation of norepinephrine varies with adrenergic drug concentration.

CHAPTER V:

FINAL THOUGHTS

In conclusion, the results of this project suggest that the sympathetic nervous system can act to modulate the production of tumor necrosis factor-α and transforming growth factor-β by lymphocytes and alveolar macrophages through the neurotransmitter norepinephrine. Although the interpretation of this research thesis suggests the mechanism of neuroimmunomodulation is primarily through the β-adrenoceptor of the lymphocyte and alveolar macrophage, the adrenoceptor pathway stimulated by norepinephrine, and thus the respective effects are debatable, and is therefore the results are open to various interpretations.

In CD3 + PMA activated murine splenocytes, NE was able to modulate a decrease in both TNF-α and TGF-β production through the β-adrenoceptor, while increases in both cytokines are observed when α-adrenergic treatments are used to target the α-adrenoceptors. These effects, although they are both statistically significant over a two day time course, only appear to be dose dependent when the β-adrenoceptor is targeted. In the non-stimulated murine splenocytes as well as in both transformed cell lines, TGF-β and TNF-α production was augmented by both the α-adrenergic agonists and β-adrenergic agonists over the two day time course. Although only the β-adrenergic agonists had a dose dependent effect on the production of cytokines, the α-adrenergic agonists were able to increase TGF-β and TNF-α to larger quantities than the β-adrenergic treatments. The production of biologically active TGF-β was found predominately in the secretions of the CD3 + PMA stimulated population murine splenocytes as well as the two transformed cell lines; the non-stimulated murine splenocytes produced predominately latent TGF-β.

In this study, norepinephrine was also able to significantly augment LPS-stimulated alveolar macrophage production of both TGF-β and TNF-α over a two day

time course. The results illustrate that when this modulation occurred through the β-adrenoceptor of the LPS stimulated murine alveolar macrophage, a significant decrease in the production of total TGF-β was observed, while a significant increase in total TGF-β production was observed when a β-adrenergic antagonist was utilized in conjunction with NE. The biological activity of the TGF-β secreted by the alveolar macrophages suggests that LPS stimulation results in the production of active TGF-β, while a lack of LPS stimulation relates to increased latent TGF-β production.

One major drawback throughout this study was the heavy reliance on the MTT method for quantification of both TGF-β and TNF-α. Although this method of quantification is acceptable, it is imperative that the results of these experiments be verified for their accuracy utilizing another biological assay, such as the ELIZA or radioimmunoassay method. Utilizing either of these classical methods to verify the results of theses studies would strengthen the results of this project and would also prove that the measured cytokines -- TGF-β and TNF-α -- were indeed the cytokines modulated by norepinephrine.

Although the original study for this project was performed over a three day time course, only 24 hr and 48 hr data is included in this thesis. Because, the half life of all the agonist treatments is short, the possibility of these drugs having an effect at the 72 hr time point is very unlikely, and thus it was felt that this data could be safely eliminated. It can also be postulated that 48 hr data obtained for this project may not be justified, again due to the short half lives of the adrenergic drugs, however, this data was included to examine the effects of the various treatments on TNF-α and TGF-β production over time.

Finally, in many of the experiments, the range of change for many of the results is quite small, and thus it is difficult to determine, especially from the studies performed, whether or not these effects are biologically significant. Therefore, it is imperative that more studies, especially *in vivo* studies, are performed to examine if

these small changes in cytokine production, modulated by NE, are of biological significance. Also, although the results in this thesis are consistent throughout, the cell lines were never tested for mycoplasma, and therefore before this data can be accepted as true, a screening process of the various cell lines must be performed to guarantee that it was in fact the tested cell lines and not mycoplasma providing the observed conclusions.

The preliminary results of this project illustrate the novel yet complex interactions which occur between the nervous and immune systems. This study has demonstrated the modulation of lymphocyte and alveolar macrophage derived TNF-α and TGF-β by the adrenergic agonist norepinephrine, and has thus defined, although only in a rudimentary level, one of the many processes of neuroimmunomodulation known to play important roles in the pathogenesis of many immunologically mediated diseases, including idiopathic pulmonary fibrosis, tuberculosis, sarcoidosis and chronic lung disease of the premature infant.

APPENDIX A

MATERIALS

MEDIA AND MATERIALS FOR TISSUE CULTURE

b-mercaptoethanol (2-hydroxyethyl mercaptan)
: Sigma Chemical Co. Cat.#M6250
: F.W. 78.13
: concentration utilized in culture media was 5.0×10^{-5} M

gentamycin sulphate
: Sigma Chemical Co. Cat.#G3632
: concentration utilized in cell culture media was 5 mg/mL

D-glucose, anhydrous
: BDH Chemicals Cat.#B28450
: F.W. 180.16
: concentration utilized in cell culture media for WEHI164var and A375 was 4.5 g/L

L-glutamine
: Sigma Chemical Co. Cat.#G1251
: F.W. 146.15
: $C_5H_{10}N_2O_3$
: concentration utilized in cell culture media was 2.0×10^{-3} M

sodium pyruvate
: Sigma Chemical Co. Cat.#S8636
: F.W. 110.04
: concentration utilized in cell culture media was 1.0×10^{-3} M.

trypsin-EDTA
: Gibco Laboratories Cat.#15400-054
: 0.5% Trypsin, 5.3 mM EDTA.4Na
: concentration utilized for removing adherent cells from tissue culture flasks
1:10 for WEHI-16VAR
1:2 for A375
- trypsin-EDTA was diluted in RMPI-1640PR

TISSUE CULTURE MEDIA

RPMI-1640 with L-glutamine and phenol red, without glucose and sodium bicarbonate
: Gibco Laboratories Cat.#31800-071

For EL-4, Jurkat and 145-2C11 cell lines:

Complete culture media:	RPMI-1640 with 10% FBS, 1.0 x 10-3 M sodium pyruvate, 2.0 x 10-3 M L-glutamine, 5 mg/mL gentamycin sulfate
Experimental media:	RPMI-1640 with 10% FBS, 1.0 x 10-3 M sodium pyruvate, 2.0 x 10-3 M L-glutamine, 5 mg/mL gentamycin sulfate

For A375 and WEHI-13VAR cell lines:

Complete culture media:	RPMI-1640 with 10% FBS, 1.0 x 10-3 M sodium pyruvate, 2.0 x 10-3 M L-glutamine, 5 mg/mL gentamycin sulfate and 4.5 mg/mL glucose
Experimental media	RPMI-1640 with 10% FBS, 1.0 x 10-3 M sodium pyruvate, 2.0 x 10-3 M L-glutamine, 5 mg/mL gentamycin sulfate and 4.5 mg/mL glucose

TISSUE CULTURE SERA

Controlled Process Serum Replacement-1 (CPSR-1)
: Sigma Chemical Co. Cat.#C8905

Newborn Calf Serum
: Sigma Chemical Co. Cat.#N4637

Serumax Fetal Bovine Serum
: Sigma Chemical Co. Cat.#S8894

TISSUE CULTURE CONTAINERS

24 wells/plate, rigid polystyrene plates, tissue culture treated
: Sigma Chemical Co. Cat.#M9655

96 wells/plate, rigid polystyrene plates, tissue culture treated
: Sigma Chemical Co. Cat.#M9780

25 cm^2 canted neck with plug cell culture flasks, tissue culture treated
: Sigma Chemical Co. Cat.#C7064

75 cm^2 canted neck with screw top cell culture flasks, tissue culture treated
: Sigma Chemical Co. Cat.#C0427

CYTOKINES

tumor necrosis factor-α
: Peprotech Cat.#315-O1A
: recombinant murine TNF-a

anti-tumor necrosis factor-α
: Peprotech Cat.#500-P6A
: anti-murine TNF-a

transforming growth factor-β
: R & D
: Cat.#240-B
: recombinant human TGF-β

anti-transforming growth factor-β
: R & D
: Cat.#AF-101NA
: polyclonal neutralizing antibody

CELL LINES

EL-4 : T cell, mouse (ATCC TIB39)

JURKAT : lymphoma, Jurkat derivative, human (ATCC CRL8163)

WEHI-13VAR : fibrosarcoma, mouse (ATCC CRL2148)

A375 : malignant melanoma, human (ATCC CRL1619)

145-2C11 : hybridoma, cultures secrete mAB specific for murine CD3 epsilon chain
: murine (ATCC CRL1975)

PHARMACOLOGIC REAGENTS

L(-)-Norepinephrine bitartrate
: Sigma Chemical Co. Cat.#A9512
: (-)-arterenol
: α_1, α_2, β agonist
: adrenergic neurotransmitter, vasoconstrictor
: bitartrate salt
: F.W. 319.27
: $C_8H_{11}NO_3*C_4H_6O_6$

Terbutaline
: Sigma Chemical Co. Cat.#T2528
: (2-t-Butylamino-1-[3,5-dihydroxyphenyl]ethanol)
: β_2 adrenoceptor agonist
: hemisulfate salt
: F.W. 274.3
: $C_{12}H_{19}NO_3*1/2H_2SO_4$

UK14304
: generous gift of A. McNichol, University of Manitoba
: (5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine)
: α2 agonist
: F.W. 442.3
: $C_{11}H_{10}BrN_5$

Propranolol
: Sigma Chemical Co. Cat.#P0884
: (1-[isopropylamino]-3-[1-naphthyloxy]-2-propanol)
: β antagonist
: hydrochloride
: F.W. 295.8
: $C_{16}H_{21}NO_2*HCl$

Phentolamine
: Sigma Chemical Co. Cat.#P7547
: (2-[N-(m-hydroxyphenyl)-p-toluidinomethyl]-imidazoline
: α antagonist
: hydrochloride
: F.W. 317.8
: $C_{17}H_{19}N_{30}*HCl$

Methoxamine
: Sigma Chemical Co. Cat.#M6524
: (a-[1-amnoethyl]-2,5-dimethoxybenzyl alcohol)
: α_1 agonist
: hydrochloride
: F.W. 247.7
: $C_{11}H_{17}NO_3$*HCl

Lipopolysaccharide
: Sigma Chemical Co. Cat.#L4391
: from E. coli 0111:84
: stimulation index 12.2 @ 15.6 ug/mL LPS

PROTEASE INHIBITORS

Aprotinin
: Sigma Chemical Co. Cat.#A3428
: F.W. 6,500
: dissolved in phosphate buffered saline
: used at a final concentration of 1 ug/mL
: inhibits: trypsin, chymotrypsin, kallikrein, plasmin

Leupeptin
: Sigma Chemical Co. Cat.#L2023
: F.W. 475.6
: dissolved in distilled, de-ionized water
: used at a final concentration of 0.5 ug/mL
: inhibits: acid proteases

Pepstatin A
: Sigma Chemical Co. Cat.#P4265
: F.W. 685.9
: dissolved in methanol
: used at a final concentration of 0.5 ug/mL
: inhibits: acid proteases such as pepsin, renin, cathepsin D

CHEMICALS

ammonium chloride : Fisher Scientific Cat.#A661
: NH_4Cl
: F.W. 53.5

ammonium sulphate : BDH Chemicals Cat.#ACS093
: $(NH_4)_2SO_4$
: F.W. 132.14

bovine serum albumin : Sigma Chemical Co. Cat.#A3350
: Fraction V
: 96-99% Albumin

dimethyl sulfoxide : Fisher Scientific Cat.#D128-500
: $[CH_3]_2SO$
: F.W. 78.13

erythrocyte lysis buffer or tris-ammonium chloride buffer
: TRIZMA base (Tris[hydroxymethyl]aminomethane
: Sigma Chemical Co. Cat.#T6791
: F.W. 121.5
: Stock solution was 0.17M @ pH 7.65
: buffer: 0.017 M Tris, 0.144 M NH_4Cl @ pH2

hepes : Sigma Chemical Co. Cat.#H9136

hydrochloric acid : Sigma Chemical Co. Cat.#H7020
: F.W. 36.46
: HCl

MTT : Sigma Chemical Co. Cat.#M5655
: F.W. 414.3
: (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)
: used at a final experimental concentration of 5 mg/mL

phosphate buffered saline (PBS)
: 0.14 M NaCl, 0.00015 M KH_2PO_4, 0.0005 M $NaHPO_4$, 0.0004 KCl
: pH 7.2

potassium chloride : Fisher Scienctific Cat.#P217
: F.W. 74.56
: KCl

potassium phosphate monobasic
: Aldrich Chemical Co. Cat.#22130-9
: F.W. 136.09
: KH_2PO_4

sigmacote : Sigma Chemical Co. Cat.#SL-2
: all glassware which came in contact with TGF-b received a pre-coating with sigmacote

sodium bicarbonate : Fisher Scientific Cat.#S233
: F.W. 84.01
: $NaHCO_3$

sodium chloride : Fisher Scientific Cat.#S2711
: F.W. 58.44
: NaCl

sodium hydroxide : BDH Chemical Co. Cat.#ACS816
: F.W. 40.00
: NaOH

sodium phosphate dibasic
: J. T. Baker Chemical Co. Cat.#3824
: F.W. 141.96
: Na_2HPO_4

solution D : 500 uL Hepes + 200 uL 5M NaOH)

trypan blue: : Sigma Chemical Co. Cat.#T0776
: F.W. 960.8
: $C_{34}H_{24}N_6O_{14}S_4Na_4$
: used for staining cells for counting and determining cell viability

EQUIPMENT

Cambridge Technology Inc. Plate reader comptuer compatible software

Cambridge Technology Inc. Plate reader 750, Version 2.0

Canadian Cabinets Flow Hood

IEC Centrifuge

GLOSSARY

Definitions of terms utilized throughout this thesis were obtained from two sources, each denoted by the use of a superscript. [a] denotes terms whose definitions were found in (98) and [b] denotes definitions obtained from (http://www.hti.umich.edu/dict/oed/simple.html).

adrenalectomy[a]	the surgical removal of one or both adrenal glands
adrenergic[a]	relating to epinephrine, it's release or action; acting like adrenaline
affinity[b]	the binding strength between a single receptor site and a ligand
agonist[a]	a chemical substance capable of combining with a receptor on a cell and initiating a reaction or activity
allogenic[b]	denoting members of the same species that differ genetically
antagonist[a]	a chemical substance capable of combining with a receptor on a cell and reducing the physiological activity of another chemical substance
antibody[b]	a protein consisting of two identical heavy chains and two identical light chains that recognizes a particular epitope on an antigen and facilitates clearance of that antigen
antigen[b]	any substance that binds specifically to an antibody or a T cell receptor
antigen processing cell[b]	any cell that can process and present antigenic peptides in association with class II MHC molecules and deliver a costimulatory signal necessary for T cell activation
B cell[b]	any of the lymphocytes that have antibody molecules on the surface and comprise the antibody-secreting plasma cells when mature; a lymphocyte that matures in the bone marrow and expresses membrane-bound antibody. Following interaction with antigen, it differentiates into antibody-secreting plasma cells and memory cells
β_2-microglobulin[b]	invariant subunit that associates with the polymorphic a chain to form class I MHC molecules, it is not encoded by MHC genes
carcinoma[b]	tumour arising from endodermal or ectodermal tissues

catecholamine[a]	any of various amines that function as hormones or neurotransmitters or both; a substance that transmits nerve impulses across a synapse
CD4[a]	a large glycoprotein that is found on the surface of helper T cells
CD antigen[b]	cell-membrane molecule used to differentiate human leukocyte subpopulations and identified by monoclonal antibody. All monoclonal antibodies that react with the same membrane molecule are grouped into a common cluster of differentiation, or CD
cell line[b]	a population of cultured tumor cells or normal cells that have been subjected to chemical or viral transformation. Cell lines can be propagated indefinitely in culture.
central nervous system[a]	the portion of the vertebrate nervous system consisting of the brain and the spinal cord
class I MHC molecule[b]	heterodimeric membrane proteins that consist of an α chain encoded in the MHC associated noncovalently with β_2-microglobulin. They are expressed by nearly all nucleated cells and function in antigen presentation to CD8+ T cells
class II MHC molecule[b]	heterodimeric membrane proteins that consist of a noncovalently associated α and β chain, both encoded in the MHC. They are expressed by antigen-presenting cells and function to present antigen to CD4+ T cells
cyclic AMP[a]	a cyclic mononucleotide of adenosine that is formed from ATP and is responsible for the intracellular mediation of hormonal effects on various cellular processes; also called adenosine 3', 5' - monophosphate
cytokine[b]	any of numerous secreted, low molecular weight proteins that regulate the intensity and duration of the immune response by exerting a variety of effects on lymphocytes and other immune cells
cytotoxic[b]	having the ability to kill cells
endogenous[a]	produced or synthesized within the organism or system

endotoxins[b]	certain lipopolysaccharide components of the cell wall of gram-negative bacteria that are responsible for many of the pathogenic effects associated with these organisms
exogenous[a]	introduced or produced outside the organism or system; specifically not synthesized within the organism or system
helper T cells[a]	a T cell that participates in an immune response by recognizing a foreign antigen and secreting lymphokines to activate T cell and B cell proliferation, that usually carries CD4 molecular markers on its cell surface
hematopoiesis[b]	formation and development of the red and white blood cells
hybridoma[b]	a clone of hybrid cells formed by fusion of normal lymphocytes with myeloma cells; it retains the properties of the normal cell to produce antibodies or T cell receptors but exhibits the immortal growth characteristic of myeloma cells. Hybridomas are used to produce monoclonal antibodies
immune system[a]	the bodily system that protects the body from foreign substances, cells and tissues by producing the immune response and that includes especially the thymus, spleen, lymph nodes, special deposits of lymphoid tissue, lymphocytes including the B cells, T cells and antibodies
interferon[a,b]	any of a group of heat-stable soluble basic antiviral glycoproteins of low molecular weight that are produced usually by cells exposed to the action of a virus, sometimes to the action of another intracellular parasite (as a bacterium), or experimentally to the action of some chemicals, substances which help to regulate the immune response
interleukin[a,b]	any of several compounds of low molecular weight that are produced by lymphocytes, macrophages, and monocytes and that function especially in regulation of the immune system and especially cell-mediated immunity
in vitro[a]	in an artificial environment outside the living organism
in vivo[a]	within a living organism
leukocyte[b]	any blood cell that is not an erythrocyte; white blood cell

lymph node[b]	a small secondary lymphoid organ that contains lymphocytes, macrophages and dendritic cells and serves as a site for filtration of foreign antigen and activation and proliferation of lymphocytes
lymphocyte[a,b]	an agranulocytic leukocyte that normally makes up a quarter of the white blood cell count but increases in the presence of infection; a mononuclear leukocyte that mediates humoral or cell-mediated immunity
lymphokine[b]	a cytokine produced by activated lymphocytes, especially helper T cells
macrophage[b]	a large leukocyte derived from a monocyte that functions in phagocytosis, antigen processing and presentation, secretion of cytokines, and antibody-dependent cell-mediated cytotoxicity
MHC[a]	major histocompatibility complex; a complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells and for rapid graft rejection. It is call the H-2 complex in the mouse and the HLA complex in humans
metastasis[a]	change of position, state, or form, as a transfer of a disease-producing agency from the site of disease to another part of the body, as a secondary metastatic growth of a malignant tumor
mitogen[b]	any substance that nonspecifically induces DNA synthesis and cell division, especially that of lymphocytes. A common mitogen is LPS.
monoclonal antibody[b]	homogenous preparation of antibody molecules, produced by a hybridoma, all of which exhibit the same antigenic specificity
noradrenergic[a]	liberating, activated by, or involving norepinephrine in the transmission of nerve impulses <noradrenergic nerve endings; noradrenergic nerve fibers>
norepinephrine[a]	a catecholamine, that is the chemical means of transmission across synapses in postganglionic neurons of the sympathetic nervous system and in some parts of the central nervous system, is a vasopressor hormone of the adrenal medulla, and is a precursor of epinephrine in its major biosynthetic pathway

platelet[b] — a small nuclear membrane-bound cytoplasmic structure derived from megakaryocytes, which contains vasoactive substances and clotting factors important in blood coagulation, inflammation and allergic reactions

primary lymphoid organ[b] — organs in which lymphocyte precursors mature into antigenically committed, immunocompetent cells. In mammals, the bone marrow, and thymus are the primary lymphoid organs in which B-cell and T-cell maturation occur, respectively.

secondary lymphoid organ[b] — organs and tissues in which mature, immunocompetent lymphocytes encounter trapped antigens and are activated into effector cells. In mammals, the lymph nodes and spleen, and mucosal lymphoid tissue constitute the secondary lymphoid organs

spleen[b] — secondary lymphoid organ where old erythrocytes are destroyed and blood-borne antigens are trapped and presented to lymphocytes

stem cell[b] — cell from which differentiated cells derive

sympathectomy[a] — the surgical interruption of a nerve pathway in the sympathetic nervous system

sympathetic nervous system[a] — originates in the thoracic regions of the spinal cord; opposes physiological effects of the parasympathetic: reduces digestive secretions; speeds the heart; contracts blood vessels

systemic[a] — of relating to, or common to a system: as affecting the body generally, or by supplying those parts of the body that receive blood through the aorta rather than through the pulmonary artery

T cell[b] — a lymphocyte that matures in the thymus and expresses a T cell receptor, CD3 and CD4 or CD8. Several distinct T cell subpopulations are recognized.

T cell receptor[b] — antigen binding molecule expressed on the surface of T cells and associated with CD3. It is a heterodimer consisting of either an α or γ chain or a γ or δ chain

Th1 subset[b]	subpopulation of activated CD4+ T cells that secrete characteristic cytokines and function primarily in cell-mediated responses by promoting activation of macrophages
Th2 subset[b]	subpopulation of activated CD4+ T cells that secrete characteristic cytokines and function primarily in the humoral response
thymus gland[b]	a ductless gland in the throat, or in the neighbouring region, of nearly all vertebrates that produces lymphocytes and aids in producing immunity
tumor necrosis factor[a]	a protein that is produced by monocytes and macrophages response especially to macrophages in response specially to endotoxins and that activates leukocytes and as antitumor activity

BIBLIOGRAPHY

1. **Norris, J. G. and E. N. Benveniste.** 1993. Interleukin-6 production by astrocytes: induction by the neurotransmitter norepinephrine. *J. Neuroimmunol. 45:137.*

2. **Lilly, M. P. and D. S. Gann.** 1992. The hypothalamic-pituitary-adrenal-immune axis. *Arch. Surg. 127:1463.*

3. **Friedman, E. M. and M. R. Irwin.** 1997. Modulation of immune cell function by the autonomic nervous system. *Pharmacol. Ther. 74:27.*

4. **Heijnen, C. J., C. Rouppe van der Voort, N. Wulffraat, J. van der Net, W. Kuis and A. Kavelaars.** 1996. Functional α1-adrenergic receptors on leukocytes of patients with polyarticular juvenile rheumatoid arthritis. *J. Neuroimmunol. 71:223.*

5. **Matsuoka, I., M. Meyer and J. Thoenen.** 1991. Cell-type-specific regulation of nerve growth factor (NGF) synthesis in non-neuronal cells: comparison of schwann cells with other cell types. *J. Neuroscience 11:3165.*

6. **Weigent, D. A. and J. E. Blalock.** 1995. Associations between the neuroendocrine and immune systems. *J. Leukoc. Biol. 57:137.*

7. **Leonard, B. E. and C. Song.** 1996. Stress and the immune system in the etiology of anxiety and depression. *Pharmacology, Biochemistry and Behaviour 54:299.*

8. **Kusnecov, A. W. and B. S. Rabin.** 1994. Stressor-induced alterations of immune function: mechanisms and issues. *Int. Arch. Allergy Appl. Immunol. 105*:107.

9. **Rogers, M. P. and M. Fozdar.** 1996. Psychoneuroimmunology of autoimmune disorders. *Adv. Neuroimmunol. 6:169.*

10. **Madden, K. S. and S. Livnat.** 1991. Catecholamine action and immunologic reactivity. *Psychoneuroimmunology 75:283.*

11. **Madden, K. S., V. M. Sanders and D. L. Felten.** 1995. Catecholamine influences and sympathetic neural modulation of immune responsiveness. *Annu. Rev. Pharmacol. Toxicol. 35:417.*

12. **Green-Johnson, J. M., S. Zalcman, C. Y. Vriend, S. Dolina, D. M. Nance and A. H. Greenberg.** 1996. Role of norepinephrine in suppressed IgE production in epilepsy-prone mice. *Life. Sci. 59:1121.*

13. **Buckingham, J. C., H. D. Loxley, A. D. Taylor and R. J. Flower.** 1994. Cytokines, glucocorticoids and neuroendocrine function. *Pharmacol. Res. 30:35.*

14. **Ricciardi-Castagnoli, P., L. Pirami, M. Righi, P. Sacerdote, V. Locatelli, M. Bianchi, M. Sassano, P. Valsasnini, S. Shammah and A. E. Panerai.** 1996. Cellular sources and effects of tumor necrosis factor-α on pituitary cells and in the central nervous system. *Ann. N. Y. Acad. Sci. 150:156.*

15. **Alper, J.** 1991. Interplay of the immune and nervous systems in AIDS. *ASM News 57:411.*

16. **Dardenne, M. and W. Savino.** 1996. Interdependence of the endocrine and immune systems. *Adv. Neuroimmunol. 6:297.*

17. **Straub, R. H., M. Herrmann, T. Frauenholz, G. Berkmiller, B. Lang, J. Schölmerich and W. Falk.** 1996. Neuroimmune control of interleukin-6 secretion in the murine spleen. Differential β-adrenergic effects of electrically released endogenous norepinephrine under various endotoxin conditions. *J. Neuroimmunol. 71:37.*

18. **Straub, R. H., B. Lang, W. Falk, J. Schölmerich and E. A. Singer.** 1995. *In vitro* superfusion method for the investigation of nerve-immune cell interaction in murine spleen. *J. Neuroimmunol. 61:53.*

19. **Chambers, D. A., R. L. Cohen and R. L. Perlman.** 1993. Neuroimmune modulation: signal transduction and catecholamines. *Neurochem. Int. 22:95.*

20. **Carlson, S. L., W. H. Brooks and T. L. Roszman.** 1989. Neurotransmitter-lymphocyte interactions: dual receptor modulation of lymphocyte proliferation and cAMP production. *J. Neuroimmunol. 24:155.*

21. **Vriend, C. Y., L. Zuo, D. G. Dyck, D. M. Nance and A. H. Greenberg.** 1993. Central Administration of interleukin-1b increases norepinephrine turnover in the spleen. *Brain Res. Bull. 31:39.*

22. **Sun, L. and D. Ganea.** 1993. Vasoactive intestinal peptide inhibits interleukin (IL)-2 and IL-4 production through different molecular mechanisms in T cells activated via the T cell receptor/CD3 complex. *J. Neuroimmunol. 48:59.*

23. **Fife, A., P. J. Beasley and D. L. Fertig.** 1996. Psychoneuroimmunology and cancer: historical perspectives and current research. *Adv. Neuroimmunol. 6:179.*

24. **Dhabhar, F. S., A. H. Miller, B. S. McEwen and R. L. Spencer.** 1995. Effects of stress on immune cell distribution. *J. Immunol. 154:5511.*

25. **Paul-Eugène, N., J. P. Kolb, C. Damais, A. Abadie, J. M. Menica-Huerta, P. Braquet, J. Bousquet and B. Dugas.** 1994. β2-adrenoceptor agonists regulate the IL-4-induced phenotypical changes and IgE-dependent functions in normal human monocytes. *J. Leukoc. Biol. 55:313.*

26. **Tizard, I. R.** 1995. Immunology: an introduction; fourth edition. *Saunders College Publishing: Philadelphia.*

27. **Flamand, V., D. Abramowicz, M. Goldman, C. Biernaux, G. Huez, J. Urbain, M. Moser and O. Leo.** 1990. Anti-CD3 antibodies induce T cells from unprimed animals to secrete IL-4 both *in vitro* and *in vivo*. *J. Immunol. 144:2875.*

28. **Panina-Bordignon, P. D. Mazzeo, P. Di Lucia, D. D'Ambrosio, R. Lang, L. Fabbri, C. Self and F. Sinigaglia.** 1997. β2-agoinsts prevent Th1 development by selective inhibition of interleukin 12. *J. Clin. Invest. 100:1513.*

29. **Mosmann, T. R. and S. Sad.** 1996. The expanding universe of T -cell subsets: Th1, Th2 and more. *Immunol. Today 3:138.*

30. **Elenkov, I. J., D. A. Papanicolaou, R. Wilder and G. Chrensos.** 1996. Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. *Proc. Assoc. Amer. Phys. 168:376.*

31. **Carter, L. L. and R. W. Dutton.** 1996. Type 1 and Type 2: a fundamental dichotomy for all T-cell subsets. *Curr. Op. Immunol. 8:336.*

32. **Romagnani. S.** 1997. The Th1/Th2 paradigm. *Immunol. Today 18:263.*

33. **Hu-Li, J., J. Ohara, C. Watson, W. Tsang and W. E. Paul.** 1989. Derivation of a T cell line that is highly responsive to IL-4 and IL-2 (CT.4R) and of an IL-2 hyporesponsive mutant of that line (CT.4S). *J. Immunol. 142:800.*

34. **Yang, X. and K. T. Hayglass.** 1993. Allergen-dependent induction of interleukin-4 synthesis *in vivo*. *Immunology 78:74.*

35. **Street, N. E. and T. R. Mosmann.** 1991. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *FASEB J. 5:171.*

36. **Yamamori, T. and A. Sarai.** 1994. Evolution of the IL-6/class 1B cytokine receptor family in the immune and nervous systems. *J. Physiol. 88:165.*

37. **Lømo, J., H. K. Blomhoff, K. Beiske, T. Stokke and E. B. Smeland.** 1995. TGF-β1 and cyclic AMP promote apoptosis in resting human B lymphocytes. *J. Immunol. 154:1634.*

38. **Coqueret, O., D. Demarquay and V. Langent.** 1996. Role of cyclic AMP in the modulation of IgE production from human mononuclear cells by the β2-adrenoceptor agonist, fenoterol. *Eur. Respir. J. 9:220.*

39. **Luber-Narod, J., R. Kage and S. E. Leeman.** 1994. Substance P enhances the secretion of tumor necrosis factor-α from neuroglial cells stimulated with lipopolysaccharide. *J. Immunol. 152:819.*

40. **Dornand, J., M. Bouaboula, A. D. D'Angeac, J. Favero, D. Shire and P. Casellas.** 1992. Contrasting effects of the protein kinase C inhibitor staurosporine on the interleukin-1 and phorbol ester activation pathways in the EL4-6.1 thymoma cell line. *J. Cell. Physiol. 151:71.*

41. **Sabbadini, E. and I. Berczi.** 1995. The submandibular gland: a key organ in the neuro-immuno-regulatory network? *Neuroimmunomodulation 2:184.*

42. **Zhang, K. and S. H. Phan.** 1996. Cytokines and pulmonary fibrosis. *Biol. Signals 5:232.*

43. **Lucas, C., S. Wallick, B. M. Fendly, I. Figari and M. A. Palladinot.** 1991. TGF-β: a possible immune regulator. *Clin. Appl. TGF-β 157:98.*

44. **Massagué, J., S. Cheifetz, M. Laiho, D. A. Ralph, F. M. B. Weis and A. Zentella.** 1992. Transforming growth factor-β. *Cancer Surv. 12:81.*

45. **Suzuki, K., A. Miyashita, Y. Inoue, S. Iki, J. Enomoto, Y. Takahashi and T. Takemura.** 1991. Interleukin-6-producing pheochromocytoma. *Acta. Haematol. 85:217.*

46. **Ignatowski, T. A. and R. N. Spengler.** 1995. Regulation of macrophage-derived tumor necrosis factor production by modification of adrenergic receptor sensitivity. *J. Neuroimmunol. 61:61.*

47. **Luheshi, G. and N. Rothwell.** 1996. Cytokines and fever. *Int. Arch. Allergy Appl. Immunol. 109:301.*

48. **Rieman, D. J., M. A. Anzano, G. W. Chan, T. J. Imburgia, J. A. Chan, R. K. Johnson and G. Greig.** 1992. Antagonism of TGF-α receptor binding and TGF-α induced stimulation of cell proliferation by methyl pheophorbides. *Oncology Res. 4:193.*

49. **Karmiol, S., D. G. Remick, S. L. Kunkel and S. H. Phan.** 1993. Regulation of rat pulmonary endothelial cell interleukin-6 production by bleomycin: effects of cellular fatty acid composition. *Am. J. Respir. Cell Mol. Biol. 9:628.*

50. **Sanders, V. M. and A. E. Munson.** 1985. Norepinephrine and the antibody response. *Pharmacol. Rev. 37:229.*

51. **Tortora, G. J. and N. P. Anagnostakos.** 1990. *Principles of Anatomy and Physiology: sixth edition.* E. A. Dollinger, ed. Harper and Row, New York, catecholamines and the sympathoadrenal system, p.332.

52. **Moses, H. L., E. Y. Yang and J. A. Pietenpol.** 1991. Regulation of epithelial proliferation by TGF-β. *Clin. Appl. TGF-β 157:66.*

53. **Chelmicka-Schorr, E., M. N. Kwasniewski and A. Czlonkowska.** 1992. Sympathetic nervous system modulates macrophage function. *Int. J. Immunopharmac. 14:841.*

54. **Chelmicka-Schorr, E., M. N. Kwasniewski and A. Czlonkowska.** 1992. Sympathetic nervous system and macrophage function. *Ann. N. Y. Acad. Sci. 650:40.*

55. **Coqueret, O., B. Dugas, J. M. Mencia-Huerta and P. Braquet.** 1995. Regulation of IgE production from human mononuclear cells by β2-adrenoceptor agonists. *Clin. Exp. Allergy 25:304.*

56. **Koh, W. S., K. H. Yang and N. E. Kaminski.** 1995. Cyclic AMP is an essential factor in immune responses. *Biochem. Biophys. Res. Comm. 206:703.*

57. **Sone, S., U. Yanagawa, E. Nishioka, G. Orino, A. Bhaskaran, K. Nii, K. Mizuno, Y. Heike, F. Ogushi and T. Ogura.** 1992. Interleukin-4 as a potent down-regulator for human alveolar macrophages capable of producing tumor necrosis factor-α and interleukin-1. *Eur. Respir. J. 5:174.*

58. **DeRijk, R. H., A. Boelen, F. J. H. Tilders and F. Berkenbosch.** 1994. Induction of plasma interleukin-6 by circulating adrenaline in the rat. *Psychoneuroendocrinology 19:155.*

59. **Spengler, R. N., R. M. Allen, D. G. Remick, R. M. Strieter and S. L. Kunkel.** 1990. Stimulation of α-adrenergic receptor augments the production of macrophage-derived tumor necrosis factor. *J. Immunol. 145:1430.*

60. **Sanders, V. M. and A. E. Munson.** 1984. Kinetics of the enhancing effect produced by norepinephrine and terbutaline on the murine primary antibody response *in vitro. J. Pharmacol. Ther. Exp. 231:527.*

61. **Fricchione, G. L., T. V. Bilfinger, A. Hartman, Y. Liu and G. B. Stefano.** 1996. Neuroimmunologic implications in coronary artery disease. *Adv. Neuroimmunol. 6:131.*

62. **Norman, J. G. and G. W. Fink.** 1997. The effects of epidural anesthesia on the neuroendocrine response to major surgical stress: a randomized prospective trial. *American Surgeon 63:75.*

63. **Strom, T. B. and C. B. Carpenter.** 1980. Cyclic nucleotides in immuno-suppression-neuroendocrine pharmacologic manipulation and *in vivo* immunoregulation of immunity acting via second messenger systems. *Transplant Proc. 12:304.*

64. **Ursin, H.** 1996. Stress, distress and immunity. *Ann. N. Y. Acad. Sci. 3:204.*

65. **Zwilling, B. S.** 1992. Stress affects disease outcomes. *ASM News 58:23.*

66. **Wan, W., C. Y. Vriend, L. Wetmore, J. G. Gartner, A. H. Greenberg and D. M. Nance.** 1993. The effects of stress on splenic immune function are mediated by the splenic nerve. *Brain Research Bull. 30:101.*

67. **Lotz, M., J. H. Vaughan and D. A. Carlson.** 1988. The effect of neuropeptides on production of inflammatory cytokines by human monocytes. *Science 241:1218.*

68. **Ho, S., R. T. Abraham, S. Gillis and D. J. McKean.** 1987. Differential bioassay of interleukin-2 and interleukin-4. *J. Immunol. Methods 98:99.*

69. **Gieni, R. S., X. Yang and K. T. Hayglass.** 1995. Comparison of [^{3}H]thymidine incorporation with MTT- and MTS-based bioassays for human and murine IL-2 and IL-4 analysis: tetrazolium assays provide markedly enhanced sensitivity. *J. Immunol. Methods 187:85.*

70. **Denizot, F. and R. Lang.** 1986. Rapid colorimetric assay for cell growth and survival. *J. Immunol. Methods 89:271.*

71. **Meager, A.** 1991. Assays for transforming growth factor β. *J. Immunol. Methods 141:1.*

72. **Roehm, N. W., G. H. Rodgers, S. M. Hatfield and A. L. Glasebrook.** 1991. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J. Immunol. Methods 142:257.*

73. **Ferrari, M., M. C. Fornasiero and A. M. Isetta.** 1990. MTT colorimetric assay for testing macrophage cytotoxic activity *in vitro*. *J. Immunol. Methods 131:165.*

74. **Mossman, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods 65:55.*

75. **Carmichael, J., W. G. DeGraff, A. F. Gazdar, J. D. Minna and J. B. Mitchell.** 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res. 47:936.*

76. **Chen, A. M., K. P. McKinnon and H. S. Koren.** 1985. Lipopolysaccharide (LPS) stimulates fresh human monocytes to lyse actinomycin D-treated WEHI-164 target cells via increased secretion of a monokine similar to tumor necrosis factor. *J. Immunol. 135:3978.*

77. **Arvin, B., L. F. Neville, F. C. Barone and G. Z. Feuerstein.** 1996. The role of inflammation and cytokines in brain injury. *Neurosci. Biobehav. Rev. 20:445.*

78. **Kim, J. W., W. G. Wierda and Y. B. Kim.** 1991. Immobilized IgG immune complex induces secretion of tumor necrosis factor-α by porcine alveolar macrophages. *Am. J. Respir. Cell Mol. Biol. 5:249.*

79. **Yamaguchi, M., M. Sakata, N. Matsuzaki, K. Koike, A. Miyake and O. Tanizawa.** 1990. Induction by tumor necrosis factor-alpha of rapid release of immunoreactive and bioactive luteinizing hormone from rat pituitary cells *in vitro. Neuroendocrinology 52:468.*

80. **Bagby, G. J., C. H. Lang, N. Skrepnik and J. J. Spitzer.** 1992. Attenuation of glucose metabolic changes resulting from TNF-α administration by adrenergic blockade. *Am. J. Physiol. 262:R628.*

81. **Holt, P. G.** 1986. Down-regulation of immune responses in the lower respiratory tract: the role of alveolar macrophages. *Clin. Exp. Immunol. 63:261.*

82. **Haskó, G., I. J. Elenkov, V. Kvetan and E. S. Vizi.** 1995. Differential effect of selective block of α-adrenoreceptors on plasma levels of tumor necrosis factor-α, interleukin-6 and corticosterone induced by bacterial lipopolysaccharide in mice. *J. Endocrinol. 144:457.*

83. **R & D Systems.** 1995. Transforming Growth Factor β in R & D Catalogue. Minneapolis, Minnesota. *p.177.*

84. **van der Poll, T., J. Jansen, E. Endert, H. P. Sauerwein and S. J. H van Deventer.** 1994. Noradrenaline inhibits lipopolysaccharide-induced tumor necrosis factor and interleukin 6 production in human whole blood. *Infect. Immun. 62:2046.*

85. **Elenkov, I. J., G. Haskó, K. J. Kovács and E. S. Vizi.** 1995. Modulation of lipopolysaccharide-induced tumor necrosis factor-α production by selective α- and β-adrenergic drugs in mice. *J. Neuroimmunol. 61:123.*

86. **Street, N. E. and T. R. Mosmann.** 1991. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *FASEB J. 5:177.*

87. **Strober, W., B. Kelsall, I. Fuss, T. Marth, B. Ludviksson, R. Ehrhardt and M. Neurath.** 1997. Reciprocal IFN-γ and TGF-β responses regulate the occurrence of mucosal inflammation. *Immunol. Today 18:61.*

88. **Hamblin, A. S.** 1993. Cytokines and Cytokine Receptors. London: Oxford University Press.

89. **Ramer-Quinn, D. S., R. A. Baker and V. M. Sanders.** 1997. Activated T Helper 1 and T Helper 2 cells differentially express the β-2-adrenergic receptor. *J. Immunol. 159:4857.*

90. **Sanders, V. W. and A. E. Munson.** 1984. Role of *alpha* adrenoceptor activation in modulating the murine primary antibody response *in vitro*. *J. Pharmacol. Ther. Exp. 232:395.*

91. **Khabar, K. S. A., A. Siddiqui and J. A. Armstrong.** 1995. WEHI-13VAR: a stable and sensitive variant of WEHI-164 clone 13 fibrosarcoma for tumor necrosis factor bioassay. *Immunol. Lett. 46:107.*

92. **Röllinghoff, M. and N. L. Warner.** 1973. Specificity of *in vivo* tumor rejection assessed by mixing immune spleen cells with target and unrelated tumor cells. *Proc. Soc. Exp. Biol. Med. 144:813.*

93. **Twigg, H. L., D. S. Wilkes and D. M. Soliman.** 1994. Measurement of IL-6 inhibitory activity in cultured cell supernatants and lysates. *J. Lab. Clin. Med. 124:283.*

94. **Green-Johnson, J. M. PhD.** 1990. Studies on senescent mucosal and systemic T lymphocytes: activation, cell surface receptors, subsets, and lymphokine production. *Queen's University: Microbiology and Immunology Dept.*

95. **Paul-Eugene, N., J. P. Kolb, A. Calenda, J. Gordon, J. Kikutani, T. Kishimoto, J. M. Mencia-Huerta, P. Braquet and B. Dugas.** 1993. Functional interaction between β2-adrenoceptor agonists and interleukin-4 in the regulation of CD23 expression and release and IgE production in human. *Mol. Immunol. 30:157.*

96. **Fernandez-Botran, R. and V. Vetvicka.** 1995. Methods in Cellular Immunology. Boca Raton: CRC Press.

97. **Verstuvy, A., J. Goebels, H. Sobis and M. Vandeputte.** 1993. Influence of different growth factors on a rat choriocarcinoma cell line. *Tumor Biol. 14:46.*

98. **Kuby, J.** 1997. Immunology: third edition. New York: W. H. Freeman and Company.

99. **Sanders, V. M., R. A. Baker, D. S. Ramer-Quinn, D. J. Kasprowicz, B. A. Fuchs and N. E. Street.** 1997. Differential expression of the β2-adrenergic receptor by Th1 and Th2 clones. *J. Immunol. 158:4200.*

100. **Sanders, V.** 1995. The role of adrenoceptor-mediated signals in the modulation of lymphocyte function. *Adv. Neuroimmunol. 5:283.*

101. **Miles, K., E. Chelmicka-Schorr, G. Otten and B. G. W. Arnason.** 1984. Sympathetic nervous system and immune response in mice. *Neurology 34:259.*

102. **Gu, Y. and A. Seidel.** 1996. Influence of Salbutamol and isoproterenol on the production of TNF and reactive oxygen species by bovine alveolar macrophages and calcitriol differentiated HL-60 cells. *Immunopharmacol. Immunotoxicol. 18:115.*

103. **Sanders, V. M. and A. E. Munson.** 1984. Beta adrenoceptor mediation of the enhancing effect of norepinephrine on the murine primary antibody response *in vitro. J. Pharmacol. Ther. Exp. 230:183.*

104. **Cazaux, C. A., L. Sterin-Borda, G. Gorelik and G. A. Cremaschi.** 1995. Down-regulation of β-adrenergic receptors induced by mitogen activation of intracellular signaling events in lymphocytes. *FEBS Letters 364:120.*

105. **Heilig, M., M. Irwin, I, Grewal and E. Sercarz.** 1993. Sympathetic regulation of T-helper cell function. *Brain, Behavior and Immunity. 7:154.*

106. **Fresno, M., M. Kopf and L. Rivas.** 1997. Cytokines and infectious diseases. *Immunol. Today 18:56.*

107. **Wahl, S. M.** 1994. Transforming growth factor β: the good the bad and the ugly. *J. Exp. Med. 180:1587.*

108. **Huber, D., J. Philipp and A. Fontana.** 1992. Protease inhibitors interfere with the transforming growth factor-β-dependent but not the transforming growth factor-β-dependent pathway of tumor cell-mediated immunosuppression. *Immunology 148:277.*

109. **Zhu, J., D. A. Cohen, S. N. Goud and A. M. Kaplan.** 1996. Contribution of T lymphocytes to the development of bleomycin-induced pulmonary fibrosis. *Ann. N. Y. Acad. Sci. 796:194.*

110. **Giri, S. N., D. M. Hyde and M. A. Hollinger.** 1993. Effect of antibody to transforming growth factor β on bleomycin induced accumulation of lung collagen in mice. *Thorax 48:959.*

111. **Morganti-Kossmann, M. C., T. Kossmann, M. E. Brandes, S. E. Mergenhagen and S. M. Wahl.** 1992. Autocrine and paracrine regulation of astrocyte function by transforming growth factor-β. *J. Neuroimmunol. 39:163.*

112. **Christ, M., N. L. McCartney-Francis, A. B. Kulkarni, J. M. Ward, D. E. Mizel, C. L. Mackall, R. E. Gress, K. L. Hines, H. Tian, S. Karlsson and S. M. Wahl.** 1994. Immune dysregulation in TGF-β1-deficient mice. *J. Immunol. 156:1936.*

113. **Zafra, F., D. Lindholm, E. Castren, J. Hartikka and H. Thoenen.** 1992. Regulation of brain derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *J. Neuroscience 12:4793.*

114. **Massagué, J., J. Heino and M. Laiho.** 1991. Mechanisms in TGF-β action. *Clin. Appl. TGF-β 157:51.*

115. **Federico, M. H. H., I. Snitcovsky, S. Maistro, M. L. H. Katayama, V. L. P. Figueiredo and M. M. Brentani.** 1994. Integrin receptors and TGF-β expression in chronic myeloid leukemia cells. *Brazillian J. Med. Biol. Res. 27:2267.*

116. **Khalil, N., O. Bereznay, M. Sporn and A. H. Greenberg.** 1989. Macrophage production of transforming growth factor-β and fibroblast collagen synthesis in chronic pulmonary inflammation. *J. Exp. Med. 170:727.*

117. **Wright, J. A., E. A. Turley and A. H. Greenberg.** 1993. Transforming growth factor beta and fibroblast growth factor as promoters of tumor progression to malignancy. *Crit. Rev. Oncogenesis 4:473.*

118. **Dubinett, S. M., M. Huang, S. Dhanani, J. Wang and T. Beroiza.** 1993. Down-regulation of macrophage transforming growth factor-β messenger RNA expression by IL-7. *J. Immunol. 151:6670.*

119. **Miki, N., Y. Hamamori, K. Hirata, M. Suematsu, S. Kawashima, H. Akita and M. Yokoyama.** 1994. Transforming growth factor-β potentiated α-adrenergic and stretch-induced c-*fos* mRNA expression in rat myocardial cells. *Circ. Res. 75:8.*

120. **Roberts, A. B. and M. B. Sporn.** 1992. Mechanistic interrelationships between two superfamilies: the steroid/retinoid receptors and transforming growth factor-β. *Cancer Surv. 14:205.*

121. **Kumar, R. K., R. O'Grady, S. E. Maronese and M. R. Wilson.** 1996. Epithelial cell-derived transforming growth factor-β in bleomycin-induced pulmonary injury. *Int. J. of Exp. Pathol. 77:99.*

122. **Wahl, S. M.** 1992. TGF-β in inflammation: a cause and a cure. *J. Clin. Immunol. 12:61.*

123. **Menica-Huerta, J., M. Paul-Eugene, B. Dugas, C. Petit-Frère, J. Gordon, V. Lagente, G. Cairns and P. Braquet.** 1991. Beta-2-adrenoceptor agonists up-regulate the *in vitro* $F_C$epsilon receptor II/CD23 expression on, and release from, the promonocytic cell line U937 and human blood monocytes. *Int. Arch. Allergy Appl. Immunol. 94:91.*

124. **Wu, C. Y., V. Brinkman, D. Cox, C. Heusser and G. Delespesse.** 1992. Modulation of human IgE synthesis by transforming growth factor-β. *Clin. Immunol. Immunopathol. 62:277.*

125. **Amento, E. P. and L. S. Beck.** 1991. TGF-β and wound healing. *Clin. Appl. TGF-β. 157:115.*

126. **Snapper, C. M., W. Waegall, H. Beernink and J. R. Dasch.** 1993. Transforming growth factor-β1 is required for secretion of IgG of all subclasses of LPS-activated murine B cells *in vitro. J. Immunol. 151:4625.*

127. **Dhanani, S., M. Huang, J. Wang and S. M. Dubinett.** 1994. Interferon-alpha inhibits murine macrophage transforming growth factor-beta mRNA expression. *Inflammation 18:301.*

128. **Iizuka, K., H. Sano, H. Kawaguchi and A. Kitabatake.** 1994. Transforming growth factor β-1 modulates the number of β-adrenergic receptors in cardiac fibroblasts. *J. Mol. Cell. Cardiol. 26:435.*

129. **Martinet, Y., O. Menard, P. Vaillant, J. M. Vignaud and N. Martinet.** 1996. Cytokines in human lung fibrosis. *Arch. Toxicol. Suppl. 18:127.*

130. **Raghow, R.** 1991. Role of transforming growth factor-β in repair and fibrosis. *Chest 99:61S.*

131. **Khalil, N., R. N. O'Connor, H. W. Unruh, P. W. Warren, K. C. Flanders, A. Kemp, O. H. Bereznay and A. H. Greenberg.** 1991. Increased production and immunohistochemical localization of transforming growth factor-β in idiopathic pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol. 5:155.*

132. **Flanders, K. C. and C. F. Lippa.** 1996. TGFβ2 in chronic neurodegenerative disease. *Neural Notes 11:8.*

133. **Xing, Z., G. M. Tremblay, P. J. Sime and J. Gauldie.** 1997. Overexpression of granulocyte-macrophage colony stimulating factor induces pulmonary granulation tissue formation and fibrosis by induction of transforming growth factor-β1 and myofibroblast accumulation. *Am. J. Pathol. 150:59.*

134. **Massagué, J., A. Hata and F. Liu.** 1997. TGF-β signaling through the Smad pathway. *Trends in Cell Biol. 7:187.*

135. **Laurent, G. J., R. K. Coker and R. J. McAnulty.** 1993. TGF-β antibodies: a novel treatment for pulmonary fibrosis? *Thorax 48:953.*

136. **Gauldie, J., M. Jordana and G. Cox.** 1993. Cytokines and pulmonary fibrosis. *Thorax 48:931.*

137. **New, B. A. and L. C. Yeoman.** 1992. Identification of basic fibroblast growth factor sensitivity and receptor and ligand expression in human colon tumor cell lines. *J. Cell. Physiol. 150:320.*

138. **Toossi, Z., P. Gogate, H. Shiratsuchi, T. Young and J. J. Ellner.** 1995. Enhanced production of TGF-β blood monocytes from patients with active tuberculosis and presence of TGF-β in tuberculosis granulomatous lung lesions. *J. Immunol. 154:465.*

139. **Butler, L. D., N. K. Layman, P. E. Riedl, R. L. Cain, J. Shellhaas, G. F. Evans and S. H. Zuckerman.** 1989. Neuroendocrine regulation of *in vivo* cytokine production and effects: I. *In vivo* regulatory networks involving the neuroendocrine system, interleukin-1 and tumor necrosis factor-α. *J. Neuroimmunol. 24:143.*

140. **Franko, A. J., J. Sharplin, A. Ghahary and M. H. Barcellos-Hoff.** 1997. Immunohistochemical localization of transforming growth factor β and tumor necrosis factor α in the lungs of fibrosis-prone and "non-fibrosising" mice during the latent period and early phase after irradiation. *Radiat. Res. 147:245.*

141. **Bermudez, L. E.** 1993. Production of transforming growth factor-β by *Mycobacterium avium*-infected human macrophages is associated with unresponsiveness to IFN-γ. *J. Immunol. 150:1838.*

142. **Green-Johnson, J.** 1996. CCL-64 TGF-β radioimmunoassay protocol. Personal communication: not-published.

143. **Rodemann, H. P., A. Binder, A. Burger, N. Güven, H. Löffler and M. Bamberg.** 1996. The underlying cellular mechanism of fibrosis. *Kidney Int. 49:S32.*

144. **Schwall, R. H., K. Robbins, P. Jardieu, L. Chang, C. Lai and T. G. Terrell.** 1993. Activin induces cell death in hepatocytes *in vivo* and *in vitro*. *Hepatology 18:347.*

145. **Nelson, B. J., D. Danielpour, J. L. Rossio, J. Turpin and C. A. Nacy.** 1994. Interleukin-2 suppresses activated macrophage intracellular killing activity by inducing macrophages to secrete TGF-β. *J. Leukoc. Biol. 55:81.*

146. **Strickland, D. H., T. Thepen, U. R. Kees, G. Kraal and P. G. Holt.** 1993. Regulation of T-cell function in lung tissue by pulmonary alveolar macrophages. *Immunology 80:266.*

147. **Moineau, S. and J. Goulet.** 1991. Effect of feeding fermented milks on the pulmonary macrophage activity in mice. *Milchwissenschaft 46:551.*

148. **Franke-Ullmann, G., C. Pfortner, P. Walker, C. Steinmuller, M.L. Lohmann-Matthes and L. Kobzik.** 1996. Characterization of murine lung interstitial macrophages in comparison with alveolar macrophages *in vitro*. *J. Immunol. 157:3097.*

149. **Fels, A. O. S. and A. A. Cohn.** 1986. The alveolar macrophage. *J. Appl. Physiol. 60:353.*

150. **Yanagawa, H., S. Sone, K. Sugihara, K. Tanaka and T. OGura.** 1991. Interleukin-4 downregulates interleukin-6 production by human alveolar macrophages at protein and mRNA levels. *Microbiol. Immunol. 35:879.*

151. **Khalil, N., C. Whitman, L. Zuo, D. Danielpour and A. H. Greenberg.** 1993. Regulation of alveolar macrophage transforming growth factor-β secretion by corticosteroids in bleomycin-induced pulmonary inflammation in the rat. *J. Clin. Invest. 92:1812.*

152. **Bingisser, R., C. Stey, M. Weller, P. Groscurth, E. Russi and K. Frei.** 1996. Apoptosis in human alveolar macrophages is induced by endotoxin and is modulated by cytokines. *Am. J. Respir. Cell Mol. Biol. 15:64.*

153. **Dugas, B., N. Paul-Eugene, O. Coqueret, J. M. Mencia-Huerta and P. Braquet.** 1992. Possible role of CD23 in allergic disease. *Rev. Immunol. 143:448.*

154. **Bienkowski, R. S. and M. G. Gotkin.** 1995. Control of collagen deposition in mammalian lung. *P. S. E. B. M. 209:118.*

155. **Kotecha, S., L. Wilson., A. Wangoo, M. Silverman and R. J. Shaw.** 1996. Increase in Interleukin (IL)-1β and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity. *Pediatr. Res. 40:250.*

156. **Vaillant, P., O. Menard, J. M. Vignaud, N. Martinet and Y. Martinet.** 1996. The role of cytokines in human lung fibrosis. *Monaldi Arch. Chest Dis. 51:145.*

157. **Zhang, M., M. Gharaee-Kermani, B. McGarry, D. Remick and S. H. Phan.** 1997. TNF-α-mediated lung cytokine networking and eosinophil recruitment in pulmonary fibrosis. *J. Immunol. 158:954.*

158. **Khalil, N. and A. H. Greenberg.** 1991. The role of TGF-β in pulmonary fibrosis. *CIBA- Found - Symp. TGF-β:194.*

159. **Santana, A., B. Saxena, N. A. Nobel, L. I. Gold and B. C. Marshall.** 1995. Increased expression of transforming growth factor-β isoforms (β1, β2, β3) in bleomycin-induced pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol. 13:34.*

160. **Broekelmann, T. J., A. H. Limper, T. V. Colby and J. A. McDonald.** 1991. Transforming growth factor β1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc. Natl. Acad. Sci. USA 88:6642.*

161. **Phan, S. H., M. Gharaee-Kermani, F. Wolber and U. S. Ryan.** 1991. Stimulation of rat endothelial cell transforming growth factor-β production by bleomycin. *J. Clin. Invest. 87:148.*

162. **Silvera, M. R., G. D. Sempowski and R. P. Phipps.** 1994. Expression of TGF-β isoforms by Thy-1+ and Thy-1- pulmonary fibroblast subsets: evidence for TGF-β as a regulator of IL-1-dependent stimulation of IL-6. *Lymphokine and Cytokine Res. 13:277.*

163. **Bagchi, A., R. M. Viscardi, V. Taciak, J. E. Ensor, K. A. McCrea and J. D. Hasday.** 1994. Increased activity of interleukin-6 but not tumor necrosis factor-α in lung lavage of premature infants is associated with the development of bronchopulmonary dysplasia. *Pediatr. Res. 36:244.*

164. **Foley, N. M., A. B. Millar, A. Meager, N. M. Johnson and G. A. W. Rook.** 1992. Tumor necrosis factor production by alveolar macrophages in pulmonary sarcoidosis and tuberculosis. *Sarcoidosis 9:29.*

165. **Kotecha, S.** 1996. Cytokines in chronic lung disease of prematurity. *Eur. J. Pediatr. 155:S14.*

166. **Jagirdar, J., R. Begín, A. Durfresne, S. Goswami, T. C. Lee and W. N. Rom.** 1996. Transforming growth factor-β (TGF-β) in silicosis. *Am. J. Respir. Crit. Care Med. 154:1076.*

167. **Denis, M. and D. Bisson.** 1995. Antigen-induced alveolitis: cytokine production in a mouse model. *Inflammation 19:157.*

168. **Denis, M., D. E. Kelly and D. F. Biggs.** 1994. Release of monokines by pulmonary macrophages following antigen challenge in sensitized guinea pigs. *Inflammation 18:349.*

169. **Severn, A., N. T. Rapson, C. A. Hunter and F. Y. Liew.** 1992. Regulation of tumor necrosis factor production by adrenaline and β-adrenergic agonists. *J. Immunol. 148:3441.*

170. **Khalil, N., R. N. O'Connor, K. C. Flanders and H. Unruh.** 1996. TGF-β1, but not TGF-β2 or TGF-β3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. *Am. J. Respir. Cell Mol. Biol. 14:131.*

171. **Toulmond, S., P. Parnet and A. C. E. Linthorst.** 1996. When cytokines get on your nerves: cytokine networks and CNS pathologies. *TINS 19:409.*

172. **Nakamura, A., T. Kohsaka and E. J. Johns.** 1996. Neuro-regulation of interleukin-6 gene expression in the spontaneously hypertensive rat kidney. *J. Hypertens. 14:839.*

173. **Dunn, A. J.** 1992. The role of interleukin-1 and tumor necrosis factor α in the neurochemical and neuroendocrine response to endotoxin. *Brain Research Bull. 29:807.*

174. **Hu, X., E. A. Goldmutz and C. F. Brosnan.** 1991. The effect of norepinephrine on endotoxin-mediated macrophage activation. *J. Neuroimmunol. 31:35.*

175. **Chambers, D. A., R. L. Cohen and R. L. Perlman.** 1993. Neuroimmune modulation: signal transduction and catecholamines. *Neurochem. Int. 22:95.*

176. **Ford, J., S. Smith, J. C. Luo, G. Friedman-Jimenez, P. Brandt-Rauf, S. Markowitz, K. Garibaldi and H. Niman.** 1992. Serum growth factors and oncoproteins in firefighters. *Occup. Med. 42:39.*

177. **Spengler, R. N., S. W. Chensue, D. A. Giacherio, N. Blenk and S. L. Kunkel.** 1994. Endogenous norepinephrine regulates tumor necrosis factor-α production from macrophages *in vitro. J. Immunol. 152:3024.*

178. **Denis, M.** 1992. Interleukin-6 in mouse hypersensitivity penmonitis: changes in lung free cells following depletion of endogenous IL-6 or direct administration of IL-6. *J. Leukoc. Biol. 52:197.*

179. **Loughlin, A. J., M. N. Woodroofe and M. L. Cuzner.** 1993. Modulation of interferon-γ-induced major histocompatibility complex class II and Fc receptor expression on isolated microglia by transforming growth factor-β1, interleukin-4, noradrenaline and glucocorticoids. *Immunology. 79:125.*

IMAGE EVALUATION
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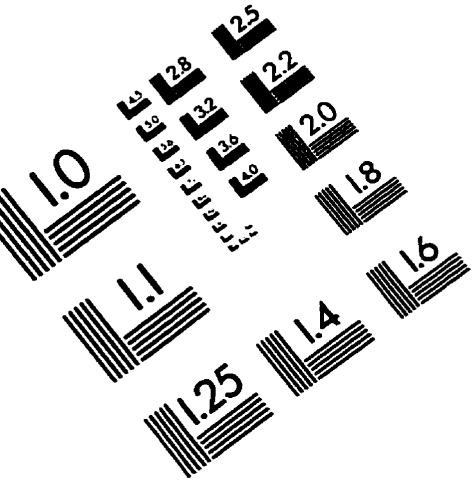

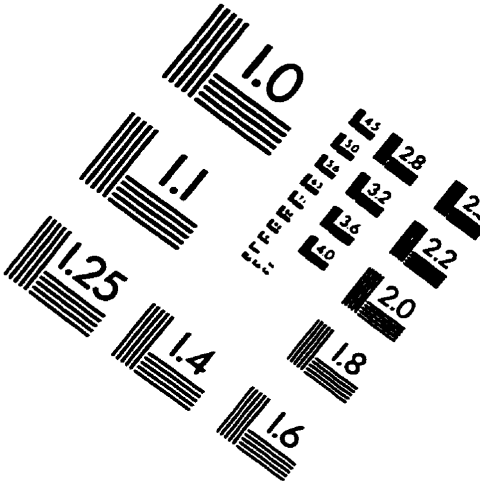

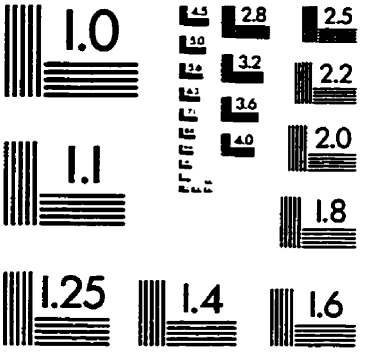

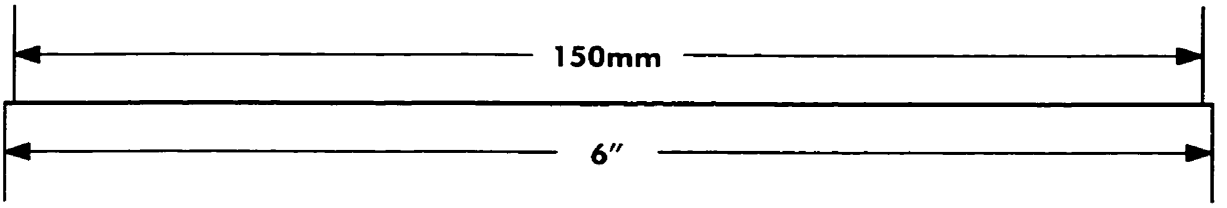

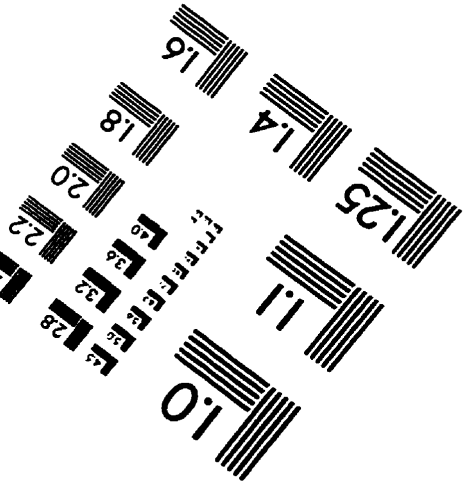

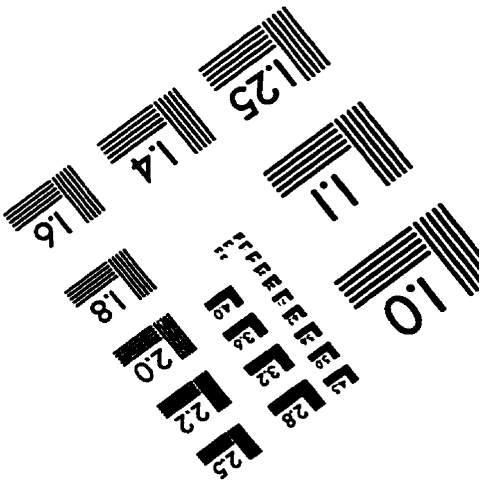

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