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Cytokine Expression by T. Helper Cells Responding to Stress

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Abstract

Cytokine Expression By T Helper Cells Responding To Stress

By Dina Zanetti

A thesis submitted in fulfillment of the requirements for the degree of Master of Science in Biology.

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Stress causes alterations to the immune system. These alterations have lead to increased susceptibility to antigens. The present study explores shifts in T helper (TH) cell balance (TH1 and TH2), through their cytokine expression. This study was performed in three phases, and focused mainly on animals subjected to a 10 day a chronic unpredictable stress paradigm. Phase one used ELISAs to confirm the cytokine profile produced from TH1 and TH2 murine cell-lines. Phase two used SDS PAGE and Western Immunoblotting to show that splenocytes from Long Evan's Hooded rats subjected to the stress paradigm, had decreased in intercellular in IL-4. Phase three used ELISAs to show inhibition to IL-10 secretion in response to stress, with additional alteration noted in IFN_Y secretion in response to isolation. These results suggest that stress and isolation cause changing cytokine secretion profiles by T helper cells, which alters the immune response.

I certify that I have read this thesis and find that, in scope and quality, it satisfies the requirement for the degree of Master of Arts/Science.

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CYTOKINE EXPRESSION BY T HELPER CELLS RESPONDING TO STRESS

By

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List Of Abbreviations

Ab	antibody
ADX	adrenalectomy
Ag	antigen
AIDS	Acquired Immune Deficiency Syndrome
APC	antigen presenting cells
BCIP	5-bromo-4-chloro-3-indolyl phosphate
CK	cyanoketone
Con A	concanavalin A
CORT	corticosterone
CRH	corticotrophin-releasing hormone
CTLs	cytotoxic T cell
CUSP	Chronic Unpredictable Stress Paradigm
DMSO	dimethyl sulfoxide
DTH	delayed type hypersensitivity
ELISA	Enzyme-Linked Immunosorbent Assays
HIV	Human Immunodeficiency Virus
HPAA	hypothalamic pituitary adrenal axis
IFN_{γ}	$interferon_{\gamma}$
Ig	immunoglobulin

IL	interleukin
MHC	major histocompatibility complex
NK	natural killer cells
NBT	nitro blue tetrazolium
SRBC	sheep red blood cells
TBS	tris buffered saline
TBST	TBS with 0.1% Tween 20
TCR	T cell receptor
CTL	cytotoxic T cell
TH	T helper cell
TH1	T helper 1 cell
TH2	T helper 2 cell
TNF_{β}	tumor necrosis factor$_{\beta}$

Introduction

Stress and its effects on the human body have become a forerunner for research in the last decade. Stress in all forms has been shown to cause alterations in normal cell function and metabolism, so much so that stress-borne diseases have become extremely prevalent in modern society. For example, stress related abnormalities can lead to gastrointestinal dysfunction, heart disease, delayed wound healing, infection susceptibility and mental illness (Kiecolt-Glaser et al., 2003).

The Immune System

The immune system serves to protect the integrity of the host, by providing defense mechanisms against invading pathogens which may cause harm. Pathogens may take the form of viruses, bacteria, and other parasites or infectious agents. In addition to foreign pathogens entering the body, the immune system is also on alert for host cells which have autoimmune qualities as well as those cells that exhibit uncontrolled growth, i.e. tumor cells. Therefore, cells of the immune system patrol the host in order to protect the host organism and to maintain homeostasis (Padgett et al., 1998).

The first line of defense against invading pathogens is the skin and other epithelial layers. These surfaces represent an impenetrable barrier to most microorganisms. If these barriers are somehow penetrated, then the immune system protects the host by initiating two distinct types of responses. The first components that the invading microorganisms encounter are part of the **innate**, or non-specific, immune system (Kohm & Sanders, 2001). Innate reactions include phagocytosis by macrophages, triggering of the alternative pathway of the complement cascade, and killing of infected cells by natural killer (NK) cells. To trigger these responses, preformed receptors on the immune cells recognize a broad range of microbes. For example, during phagocytosis macrophages engulf and internalize microorganisms that adhere to their cell surface. Once internalized, the microbes are degraded within lysosomal compartments. Macrophages also release proteins, called cytokines, to signal other immune cells to join the fight against the invading pathogen (Ziegler & Unanue, 1981). A second function of macrophages is to act as antigen presenting cells or APCs. After degradation of the pathogen by the macrophage (APC), small microbial peptides are displayed on their cell surface. Recognition of the microbial peptides by other immune cells in the immediate vicinity causes the immune response to build (Ziegler & Unanue, 1981, Razi-Wolf et al., 1992). Innate immunity is also comprised of the complement protein cascade present in the blood. Complement activation is a unidirectional series of enzymatic and

biochemical protein cleavages that occurs in the presence of a pathogen. During the innate response initiation of this cascade is stimulated by the complement proteins binding directly to the surface of a pathogen, or by binding to injured or ischemic tissue. This binding is referred to as opsonization and it increases the likelihood that the pathogen will be phagocytosed. Most microorganisms are recognized and destroyed within a few hours by the innate immune system (Ziegler & Unanue, 1981). A microbe's ability to resist or evade the mechanisms involved in innate immunity is related to its degree of pathogenicity.

Many of the components of innate immunity serve as potent mediators of the inflammatory response. Inflammation is a response to local tissue damage or infection that is characterized by redness, heat swelling and pain. The response is regulated by numerous mediators and involves the controlled migration of various leukocytes (phagocytic cells) to the site of infection. As the phagocytes destroy the invading pathogen they release lytic enzymes which may damage healthy tissue as well.

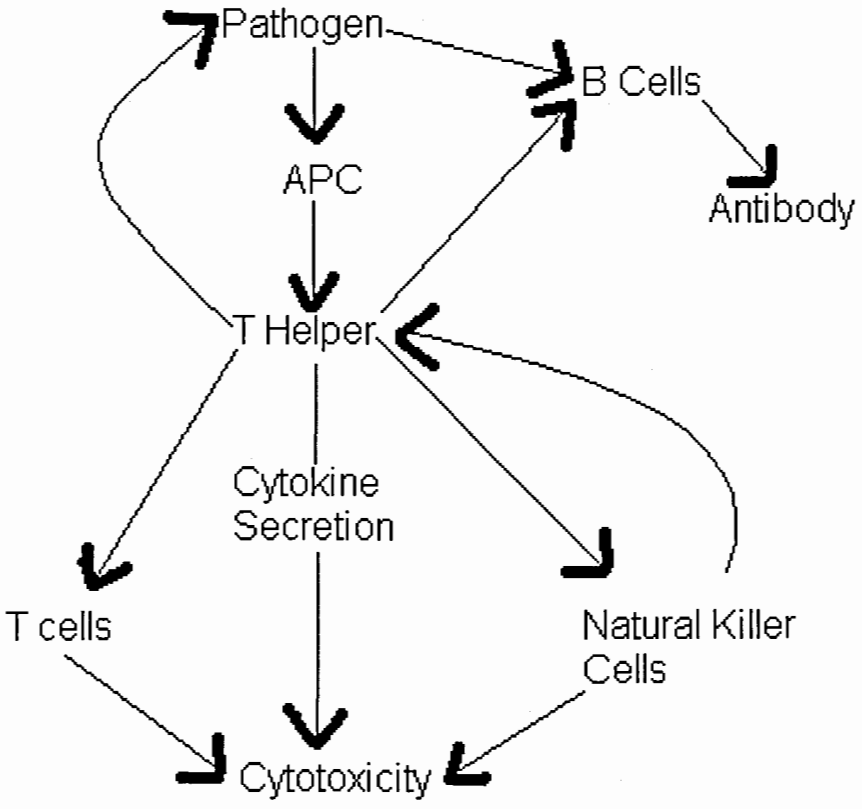
The second type of immune response is known as specific or **acquired immunity.** This branch of the immune system is specific for the particular pathogen that is causing the infection. Therefore, the main characteristic of acquired immunity is the ability to mount an exquisitely specific response to

invasion by a pathogen (Kohm & Sanders, 2001). A second characteristic of acquired immunity is the formation of memory by the immune system to the particular pathogen. Memory combined with specificity, allows each subsequent immune response to become stronger, faster and more specific (Watkins & Maier 1999).

Different pathogens require combinations of immune responses in order for that pathogen to be cleared efficiently. T-cells are responsible for **cell-mediated immunity** and B-cells are involved in antibody (Ab) production or **humoral immunity** (Abbas et al., 1996). Cytokines which are produced by immune cells and link the two branches of the immune response together. However, different infections stimulate one branch of the response more strongly than the other branch. For example, extracellular pathogens, such as bacteria, activate plasma cells (differentiated B-cells) to secrete antibodies. Antibodies are the main mediators of the humoral immune response and by binding to a pathogen they increase the likelihood that the pathogen will be destroyed by phagocytosis (Aebischer & Stadler, 1996). Antibodies bound to the surface of a pathogen will also activate complement proteins that may initiate complement-mediated lysis. In contrast, T-cells are responsible for cell-mediated immunity which destroys intracelluar pathogens, such as viruses. T-cells recognize antigens (small peptides from the pathogen), which are presented on an infected

cell's surface in association with the major histocompatibility complex (MHC) of the host cell (Ziegler & Unanue, 1981, Mossman et al., 1986, 1991). In response to this recognition, T-cells either secrete cytokines to activate other immune cells, and are therefore called **T helper (TH) cells**, or directly lyse the virally-infected cell and are referred to as **T cytotoxic cells, or CTLs** (Figure 1). CTLs are also responsible for lysing abnormal host cells such as tumor cells.

Figure 1 Flow chart of adaptive immunity. (Clancy 1998)



T helper and T cytotoxic cells can be distinguished from one another by the presence of either **CD4** or **CD8** membrane glycoproteins on their surfaces. T-cells displaying CD4 generally function as TH cells, whereas those displaying CD8 generally act as CTLs. The T helper cells can be further divided into **T helper 1 cells (TH1)** and **T helper 2 cells (TH2)** (Mossman et al., 1986). A third classification of T helper cells, **TH0**, is also known to exist. This population exhibits characteristics of both TH1 and TH2 cells. Some believe that TH1 and TH2 populations arise from TH0 cells (Mossman et al., 1991, Abbas et al., 1996) but this hypothesis has been difficult to prove and remains speculative. Classes of T helper cells are morphologically identical. The only distinguishing characteristics between these populations are the cytokines they secrete in response to an antigen challenge (Mossman et al., 1986).

The TH1 cells characteristically secrete cytokines such as interleukin-2 (IL-2), interferon$_\gamma$ (IFN$_\gamma$) and tumor necrosis factor$_\beta$ (TNF$_\beta$). IL-2 is a growth factor that induces CTL and NK cell proliferation. IFN$_\gamma$ activates macrophages and influences B-cells to produce the IgG class of antibodies which are involved in the opsonization of bacteria and viruses. TNF$_\beta$ activates primed macrophages and NK cells. These factors are responsible for many cell-mediated immune responses and play a major role in clearing viral infections (Mossman, 1986, Sompayrac, 1999). This cytokine profile is also associated with delayed type

hypersensitivity (DTH) responses. DTH is a localized inflammatory skin reaction that recruits nonspecific inflammatory cells, such as macrophages, but results in a delay in symptom presentation (Dhabhar &McEwen, 1999). A classic example of DTH is poison ivy where a response occurs hours to days after the initial exposure.

TH2 cells characteristically produce interleukin-4, 5, 6 and 10, which induce Ab production and secretion by B-cells (Mossman et al., 1986, Abbas et al., 1996). IL-4 is a growth factor that induces B-cell proliferation and isotype switching to the IgE class of antibodies which are the major mediators of parasitic infections and allergic reactions. IL-5 is a B-cell growth factor that influences IgA production which is the class of antibody responsible for clearing of mucosal infections (Sompayrac, 1999). IL-6 promotes differentiation of B-cells into antibody secreting cells. IL-10 is known to suppress cytokine production by macrophages, down-regulate MHC class II expression and inhibit antigen presentation to T-cells. Thus, IL-10 indirectly reduces cytokine production by TH1 cells (Qin et al., 2001).

As the cytokine profiles indicate TH2 cells are classically responsible for humoral immune responses and TH1 cells are classically responsible for cell-mediated responses. However, not all cytokines are exclusive to one cell

population. Both cell types may produce a number of identical cytokines upon activation, but it is the proportion of the secreted amounts of the cytokines which define the population's profile. The TH cell populations also play a role in regulating each other by the functions of the cytokines that they produce (Abbas et al., 1996). In other words, when one arm of the immune response is high, the other is usually low because when one cell population is active the other population is inhibited (Mossman et al., 1986). Within a healthy host's body, these T helper populations maintain a dynamic balance through their cross regulation of each other (Figure 2). For example, IFN_{γ} exhibits suppression over TH2 cells, while high levels of IL-4 will negatively effect the proliferation of TH1 cells (Mossman et al., 1986, Gajewski et al., 1989, Sedar & Paul, 1994). If an environmental factor stimulates one population of TH cells, cross regulation would inhibit the other population. Stress is one factor that may influence the balance between the immune responses by disrupting the TH1/TH2 ratio.

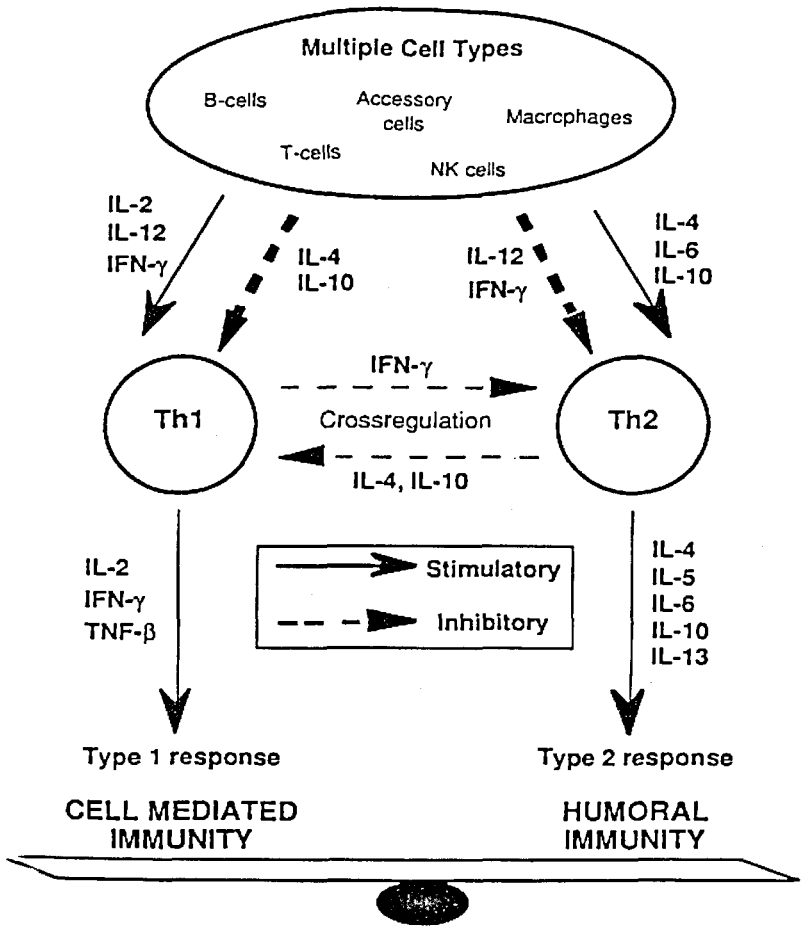


Figure 2 T helper cell balance is maintained by the cytokines released upon activation. (Kump 1998)

Stress

Stress has been defined as a constellation of events, beginning with a stimulus (stressor) that precipitates a reaction in the brain (stress perception) that subsequently activates physiologic systems in the body (stress response) (Dhabhar & McEwen, 1999). Stressors can manifest in many forms including but

not limited to psychological stress (an organism perceives an attack/harm), physiological stress (in response to the deprivation of essential needs to life, i.e. food and water), or physical stress (injury or infection). This enhanced vigilance enables an organism to understand its' environment, prepare itself to react to changes within its environment, and to adapt to the consequences of those changes. A classic example of this is a fight or flight response when the sympathetic nervous system perceives a stressful event. This perception causes a cascade of events within the body to best enable the organism to respond in the most life-preserving manner (Rosenkranz et al., 2003). The organism becomes prepared to fight for its life or to flee (Dhabhar & McEwen, 1999).

Immunologic studies demonstrate marked alterations in the immune response following a stressful event. One can assume that these altered responses are meant to prepare the organism to best react to the consequences of the stress; however, what happens when the stress does not go away? Chronic stress responses have been shown to have detrimental effects on the immune response. For example, primary care givers to incapacitated loved ones exhibit a poor antibody response to influenza vaccination and show slow wound healing. These individuals also exhibit a higher occurrence of high blood pressure and coronary disease (Kiecolt-Glaser et al., 2003). Psychological stress has also been linked to the reactivation of latent herpes simplex virus (Padgett et

al., 1998).

Stress has been shown to activate the hypothalamic pituitary adrenal axis (HPAA), ultimately producing the secretion of adrenocortical hormones, which are thought to be the primary mediators of stress effects on the immune system (Okimura, et al., 1986). This occurs when the hypothalamus releases corticotrophin-releasing hormone (CRH) which then regulates the production and release of glucocorticoids by the adrenal glands. Glucocorticoids are known to inhibit several aspects of the immune response (Morand & Leech, 1999), and therefore, have been traditionally classified as immunosuppressive hormones. For example, experiments using restrained rats demonstrate that adrenalectomy (ADX) (surgical removal of the adrenal glands) completely blocked stress-induced increases in plasma corticosterone (CORT) levels. Furthermore, the stress induced suppression of the immune response to sheep red blood cells (SRBC) challenge was not seen in these animals (Okimura et al., 1986). However, when substitute CORT was administered to ADX animals, suppression was once again noted.

Studies have also demonstrated that the release of CORT alters a number of immune responses such as leukocyte distribution (Dhabhar et al., 1996). Studies demonstrated that in blood samples from restrained male Sprague

Dawley rats there was a reduction in the presence of leukocytes coinciding with an increase in CORT levels. Researchers then treated rats with cyanoketone (CK), a steroid hormone inhibitor, which was used to block the increase in plasma CORT levels in response to the stress. Blood samples showed that CK treated animals exhibited lower CORT levels and a lack of alteration in leukocyte distribution pattern (Dhabhar et al., 1995). From these experiments (and others), we can note that CORT plays a major role in the immune system's stress response.

The release of CORT has also been shown to induce direct alterations in immune cell populations as well as to directly modulate cytokine production (Bethin, et al., 2000, Okimura, et al., 1986, Habib, et al., 2000, Watkins & Maier, 1999). CORT affects T-cell proliferation (Stefanski 1998, Stefanski & Engler, 1998) but Dhabhar et al. (1998) found that B-cells, NK cells and monocytes exhibit a greater stress-induced decrease than do T-cells. One explanation for this effect is that the release of stress hormones such as CORT increases migration of leukocytes into tissues and up-regulates adhesion factors, causing leukocytes to be retained in target tissue in preparation for an antigen challenge. TH cells are perhaps less sensitive to migration factors, however, a more in-depth explanation has not yet been offered.

The physiological response to a stressor can be relative to the degree or power of the stressor. For example, the greater the stress, the greater the response. In addition, the degree to which an organism can control the stress affects the organism's ability to cope with the event. One experiment involved rats yoked together where both animals were exposed to the same stress. One animal had control over the stress while the second animal had no control at all. Results show that animals that could control the stressor exhibited lower CORT levels than the animals that had no control (Kant et al., 1992). Another factor in the stress response seems to be socialization and stratification (Sachser & Lick, 1990). Studies in this area have shown varied responses. In some cases, the subordinate animal has shown extreme adrenal hormone increases, occasionally leading to sickness and death (Sachser & Lick, 1990). Other studies have shown the dominant animal to bear the burden of the stress response. In 1998, Stefanski and Engler studied the resident/ intruder paradigm to investigate differing immune system responses to chronic verses acute social stress in the form of social confrontation in male rats. The data collected suggests that acute social stress results in suppressed T-cell numbers and functional T-cell capacity in response to a mitogen whereas chronic stress only reduces the number of T-cells in the blood. Studies performed comparing a chronic stress immune response against an acute stress immune response support the idea that the type of stress delivery can impact the type of alterations the immune systems

responds with, for example although some immune functions can be increased after acute or short stress, long stress has an immunosuppressive effect, above all on the cellular immunity which is more susceptible to this effect than the humoral response (Millan et al., 1996). Additionally, the socialization history prior to stress initiation has been shown to alter the response to confrontation. Therefore, CORT can actually be classified as an immunomodulator, as opposed to a basic suppressor, which opens up a new avenue for research.

Purpose

The purpose of the current study is to elucidate the shift in T helper cell balance after a natural, environmental stress. First, TH1 and TH2 cell lines were subjected to varying concentrations of CORT to demonstrate that the hormone directly altered T helper cytokine profiles. Then Western blotting and Enzyme-Linked Immunosorbent Assays (ELISA) were used to analyze cytokine secretion by lymphocytes from Long-Evan's Hooded Rats (Sprague Dawley) and California Deer Mice (*Peromyscous californicus*) exposed to the Chronic Unpredictable Stress Paradigm (CUSP), a 10 day long series of mild, unpredictable stress. Chronic mild stress has been showed to cause animals physical and behavioral changes in response to the paradigm.

The significance of this research lies in a better understanding of the alterations of the immune response after exposure to chronic stress. Our current hypothesis suggests that the balance of the TH cells is altered and shifted from a TH1 to a TH2 response. However, the enhanced humoral response could be at the expense of cell mediated immunity. This same alteration in the immune response has been linked to the development of Acquired Immune Deficiency Syndrome (AIDS) in humans exposed to the Human Immunodeficiency Virus (HIV). These studies increase our understanding of these mechanisms.

The hypotheses proposed in these studies were:

1. Environmental stress causes a shift in the TH1 and TH2 cell balance.
2. The stress-induced release of CORT causes suppression of TH1 cells, which can be seen through the cytokine profile of splenocytes.
3. The suppression of cytokines produced by TH1 cells causes an increase in TH2 cell cytokine production.
4. The shift in TH1/TH2 ratios alters the overall immune response.

Materials and Methods

In vitro study

Phase One

Murine T helper 1 (TH1) cell line AE7 and T helper 2 (TH2) cell line D10.G.1 were obtained from Dr. Conrad and Dr. Tew at Virginia Commonwealth University in the Department of Microbiology and Immunology at the Medical College of Virginia campus. Cells were suspended in RPMI-1640 complete cell culture media (penicillin (10,000 U)-streptomycin (10 mg), L-glutamine (200 mM) sodium pyruvate (100 mM) all purchased from Sigma, HEPES (5 mL, 1 M, pH 7.0) 0.5 mL of 2-mercaptoethanol solution, and 10% heat-inactivated Fetal Calf Serum (HyClone SH30070.03). Sterile 48-well culture plates (Sigma) were pre-coated with 75 μl anti-CD3 (10 μg/mL in phosphate buffered saline [PBS]). Plates were gently rocked and incubated at 37°C (5% CO_2) for 3 hours and then washed three times with PBS. Each well then received 0.5 mL of suspended AE7 or D10.G.1 cells at a concentration of 5 X 10^6 cells/well. Corticosterone (CORT) was dissolved in 100% dimethyl sulphoxide (DMSO, Sigma) at a concentration of 0.1 M (34.65 mg/mL DMSO) and serial dilutions were prepared to yield various concentrations of CORT in 1% DMSO (0.01 μM, 0.10 μM, 0.50 μM, 1.0 μM, and 10 μM). Cell suspensions either received a dilution of CORT in triplicate or served as a control and received neither CORT nor vehicle. The TH cell lines

were then incubated in the anti-CD3-coated plates at 37°C (5% CO_2) for 24 hours. After incubation, plates were centrifuged for 10 minutes (500 x g) and supernatants were extracted and stored at -20°C until assayed for cytokine production.

Cell line supernatants were subjected to Quantikine ELISA analysis, using the standard ELISA protocol (R&D Systems, Minneapolis, MN). TH1 (IL-2 and IFN_Y) and TH2 (IL-4 and IL-10) cytokine secretion were each quantified for both cell lines, at all CORT concentrations.

In vivo studies

Phase Two

Subjects

In phase two of these studies twenty-five (100 - 150 grams) male Long Evan's Hooded rats were grouped into three sample sets. Eight animals were housed individually (isolates), twelve animals were housed in pairs and five animals were housed together as controls. Animals were allowed food and water ad libitum.

Animals were housed in the laboratory of Dr. Kelly Lambert at Randolph Macon College (Ashland, VA). Animals were kept in clear, plastic cages

(26.67 cm X 17.75 cm X l0.l6 cm) and kept on a 12-hour light/dark schedule. The temperature of the room was maintained at 23.1°C.

The studies were conducted over a period of ten (10) weeks. Animals were identified and body weights were recorded. Animals were allowed four (4) days to acclimate to their environment; this involved access to food and water ad libitum. On day 0, animals were subjected to an open field test (88.9cm x 76.2cm field dimensions) that involved assessing anxiety behavior. Anxiety assessment involved 4 parts:

1. observing the number of lines the animal crossed in a field grid
2. rears (how many times an animal sat back on his/her hind legs while exploring a field)
3. freezes (how many times an animal froze in place while exploring a field), latency to cross the center
4. latency to cross the center of a field, and how many times the center was crossed.

On Day 1, the Chronic Unpredictable Stress paradigm (CUSP) was implemented for the experimental/control animals (Table 1). After completion of the protocol, the animals were again exposed to the open field test. The open field tests probed the animals' behavior to see how stress affected their boldness level, curiosity and inquisitiveness. On day 11, animals were exposed to fox

urine, which is a stressor for these animals, for five minutes, and then sacrificed.

Table 1 10 Day Chronic Unpredictable Stress Paradigm

Day 1	Tilt cages at 45° angle
Day 2	Untilt cages Soil bedding with 150 mL water 7 hours white noise (90 decibels)
Day 3	Clean cages Exposure to conditioned stimulus (CS) for 15 minutes followed by 3 minute forced swim
Day 4	Tilt cages at 45° angle Exposure to strobe light (6 hours)
Day 5	Exposure to soiled bedding (cat litter) Exposure to CS, 5 minute forced swim
Day 6	Exposure to fox urine Lights off during light cycle
Day 7	Remove fox urine Exposure to acidic water (1 mL distilled vinegar in water bottle)
Day 8	Replace water bottle 6 hours strobe light Tilt cages at 45° angle
Day 9	Untilt cages Exposure to CS for 3 minutes, no forced swim
Day 10	3 hours white noise Attach tail clip to base of tail for 5 minutes Lights on during dark cycle

Phase Three

In Phase three of the study, a total of thirty-six adult male California Deer Mice (*Peromyscous californicus*) were divided into an experimental stress group (twenty) and a control group (sixteen). The experimental group was further divided into ten social animals (brothers) housed in pairs and isolated animals (ten animals housed alone). The control group was also divided into social or isolate groups. Eight mice were housed in pairs, while eight mice remained as isolates. Animals were allowed food and water ad libitum.

Studies were conducted over a ten (10) week period using the same Chronic Unpredictable Stress Paradigm used for the animals in Phase two (Table 1) with one exception; on day 10 a tail clip was added to the 10 day stress paradigm.

Perfusions

The animals were sacrificed by inhalation of methane. Once unconscious, blood was obtained from a retro-orbital bleed for glucocorticosteroid analysis. Blood was collected in heparanized capillary tubes to reduce clotting and then centrifuged for 10 minutes at 3000 x g. Serum was removed and analyzed for corticosteriods at Ohio State University. Corticosterone levels were measured using I RIA kit (ION Biochemistry). Spleens were removed and weighed. Animals

were then injected with an overdose of sodium pentobarbital. The animals were subsequently perfused for two minutes with cold PBS and then for five minutes with 4% paraformaldehyde. At the completion of the perfusion, brains were removed and placed in 4% paraformaldehyde for two hours and then in a 20% sucrose/PBS solution overnight.

Immunological Assessment

Once the spleens were harvested and weighed they were placed in RPMI-1640 complete media. Single cell suspensions were prepared by teasing cells through sterile 40 mesh sieves into RPMI – 1640 cell culture medium. Cell suspensions were washed by centrifugation at 1000 x g for 10 minutes. Supernatants were removed and splenocytes were then depleted of erythrocytes with sterile distilled H_2O and subjected to one additional wash. Cells were counted using a hemacytometer and plated in sterile 24-well culture plates, at a concentration of 2 X 10^5 cell/mL. Cells were activated with Concanavalin A (Con A) (Sigma) at 5 μg/mL for 24 hours to induce cytokine production. Cells were incubated at 37°C in 5% $C0_2$. After the incubation, cell suspensions were removed from the plate, placed in sterile microfuge tubes and centrifuged for ten (10) minutes. Supernatants were collected and stored at -70°C until assayed for cytokine production.

Supernatants from phase two samples (Long Evans Hooded Rats) were subjected to SDS-polyacrylamide gel electrophoresis in order to separate the proteins secreted from the splenocytes. A constant volume of each supernatant was added to 4X protein sample buffer and denatured at 95°C for 2-5 minutes prior to separation on a 15% acrylamide gel. Molecular weight standards were run on each gel in order to determine molecular weights of proteins found in the samples. Gels were run at 200 volts for 45 minutes. Proteins were then transferred to nitrocellulose membranes (Bio-Rad) and western immunoblotting analysis was performed. Membranes were blocked in 3% nonfat dry milk dissolved in tris buffered saline (TBS) overnight at 4°C with gentle shaking. Membranes were then rinsed in TBS and incubated with primary antibody (rabbit anti-mouse IL-2 or anti-mouse IL-4, R&D Systems) at a concentration of 0.2 μg/mL in 5 mL of TBS with 0.1% Tween 20 (TBST) and 0.2% nonfat dry milk for 1 hour at room temperature with gentle rocking. After incubation the membranes were washed 3X in TBST for 10 minutes each. Then the membranes were incubated with 1:5000 goat anti-rabbit acid phosphate conjugate secondary antibody (R&D Systems) in 5 mL TBST for 1 hour at room temperature with gentle rocking. Membranes were washed in TBST (4X) and placed in a substrate solution of 25μl nitro blue tetrazolium (NBT) and 75μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP), mixed in the dark, to induce an alkaline phosphatase color

reaction. Spot density analysis was performed to quantify the specific protein present.

Supernatants from Phase three samples (California Deer Mice) were analyzed for cytokine production by using Quantikine ELISA kits (R&D Systems) specific for IL-10, IL-4, IL-2 and IFN_Y. ELISAs were performed according to the manufacturer's instructions.

Results

Phase One

The TH1 cell line, AE7, and the TH2 cell line, D10.1 were activated to release cytokines by stimulating the T cell receptor (TCR) with an anti-CD3 antibody. The concentration of cytokines secreted by the T-cells was determined by ELISA. Table 2 demonstrates that these cells produce the expected cytokine profile after activation. As shown, cytokine secretion was inhibited in the presence of increasing CORT concentrations (data not shown).

Table 2 T cell lines subject to CORT stimulation. Cell lines show expected result, with TH1 producing quantities of IL-2 and IFN-γ, while not producing significant levels of IL-4 or IL-10. The opposite hold for the TH2 cell line.

Cytokine Secretion	T-cell lines	
	TH1 (AE7)	TH2 (D10.1)
IL-2	172.9 pg/mL	--
IFN-γ	++++	--
IL-4	--	>8000 pg/mL
IL-10	--	++++

*++++ - Above limits of quantitation

Phase Two

In order to investigate the effects of stress and social behavior on immunological responses Long Evan's Hooded rats were subjected to 10 days of chronic unpredictable stress (CUSP). Blood CORT levels were used as a known stress indicator. Table 3 demonstrates CORT levels found in control animals, isolated animals subjected to CUSP and social animals subjected to CUSP. Unexpectedly, it appeared that all animals had elevated levels of CORT, however, paired stressed animals did have lower CORT values than isolate stressed animals (although not statistically significant). Table 4 demonstrates that the individually housed stressed animals had significantly lighter spleen weights than both controls and paired stressed animals which may suggest a decreased ability to mount an immune response.

Next polyacrylamide gel electrophoresis was performed on Con A-stimulated splenocytes. Western Immunoblotting of proteins isolated from control, isolate-stressed, and social-stressed animals using an anti-IL-4 antibody underwent Densitometry analysis (Figure 3), which determined that isolate-stressed animals had less intracellular IL-4 when compared to Con A-stimulated splenocytes from paired, stressed or control animals. These findings suggest that isolate-stressed animals may secrete higher levels of IL-4 which may suppress cell-mediated immunity and delayed type hypersensitivity reactions.

Table 3 Corticosterone levels in animals housed under different conditions. Control animals and isolates show similar CORT levels. The control animals show a high CORT level, indicating that they were also stressed somehow. Paired animals showed low CORT level, indicating that something may have been mitigating the stress factor. 3-A represents control animals, 3-B represents isolated animals, and 3-C represents animals housed as pairs.

3-A	
Control animals	CORT (ng/mL)
1	314.24
2	547.07
3	221.38
4	331.87
5	437.53
Average	370.42

3-B	
Isolated Animals	CORT (ng/mL)
1	465.35
2	267.58
3	436.35
4	323.82
5	222.65
6	232.26
7	566.52
8	224.62
average	342.39

3-C	
Paired Animals	CORT (ng/mL)
1A	309.67
1B	375.21
2A	349.21
2B	125.12
3A	322.77
3B	NA
4A	366.50
4B	NA
5A	375.82
5B	308.07
6A	287.08
6B	392.81
average	321.23

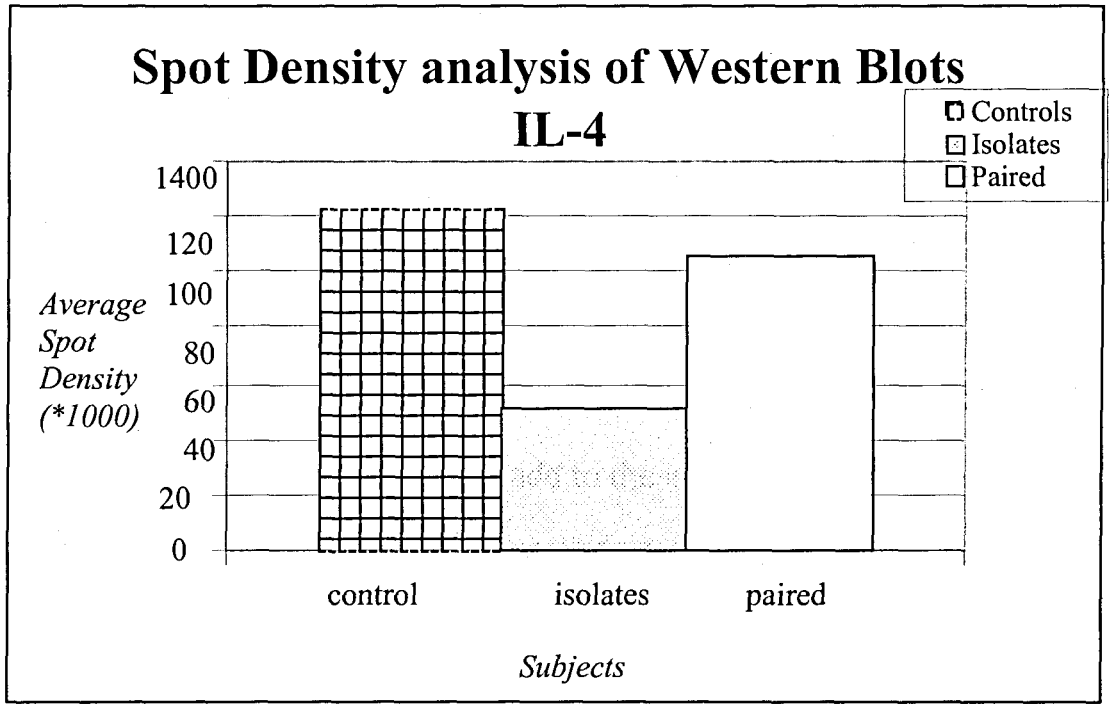
Table 4 Spleen weights from animals housed under different conditions. Isolated animals show smaller spleen weights than the control and paired counterparts, suggesting altering immune reactions to CUSP. 4-A represents control animals, 4-B represents isolated animals, and 4-C represents animals housed as pairs.

4-A	
Control Animals	Spleen weights (mg)
1	1.82
2	1.77
3	1.69
4	1.69
5	1.62
average	1.72

4-B	
Isolated Animals	Spleen weights (mg)
2	1.51
3	1.63
4	1.53
5	1.75
6	1.46
7	1.83
8	1.35
average	1.57

4-C	
Paired Animals	Spleen weights (mg)
1B	2.21
2A	1.97
2B	2.69
3A	1.63
3B	1.68
4A	2.12
4B	1.75
5A	1.63
5B	1.84
6A	1.83
6B	1.65
average	1.90

Figure 3 Spot density analysis of a random sampling of animals reveals decreased intracellular IL-4 in isolated animals. Control animals and paired animals IL-4 levels are similar.



Phase Three

In order to further analyze the effects of stress and its ability to dampen the immune response, animals were assigned to a chronic stress (isolate-stressed or social-stressed) or control group. For this phase, a new animal model was chosen, California Deer mice. This change to animal model was instilled to allow investigators to look more closely at the social behavior of the animals in addition to stress. California Deer mice are a model for social behavior. Isolation therefore would add to the investigation in some way, which was explored. After exposure of the chronic stress groups to the CUSP animals were sacrificed. CORT levels found in the different mice groups indicate that the CUSP exposure yielded animals with higher CORT levels in the blood as expected (mean 220.5). Whereas, control animals mean CORT level was 82.06 ($p=0.018$)

Splenocytes were harvested from these animals and stimulated with Con A to induce cytokine production. IL-2 and IFN_{γ} (TH1 cytokines), and IL-4 and IL-10 (TH2 cytokines) secreted from the splenocytes were analyzed by ELISA (Table 5 and Table 6). A trend for decreased IL-10 production was observed in both stress groups indicating an impact on immunological function.

Table 5 IL- 10 - Cytokine production by splenocytes from California Deer Mice. Four groups of California Deer Mice, two groups of five, and two groups of six (22) subjected to the Chronic Unpredictable Stress Paradigm. Culture concentrations were determined by ELISA and are expressed as pg/mL. Results represent pooled data per stress group.

Animal groups	# of animals producing	Average IL- 10 Production (pg/mL)
Isolates Controls	5/5	147.6
Social Controls	3/6	37.0
Isolates Stress	3/5	14.3
Social Stress	1/6	9.1

Table 6 IFN-γ – Cytokine production by splenocytes from California Deer Mice. Four groups of California Deer Mice, two Controls groups of five (5) animals each, and two experimental groups of six (6) animals each. Culture concentrations were determined by ELISA and are expressed as pg/mL. Results represent pooled data per stress group.

Groups	# of animals producing	Average IFN-γ (pg/mL)
Isolates Controls	1/5	.644
Social Controls	1/6	3.35
Isolates Stress	0/5	0
Social Stress	2/6	5.97

Discussion

Stress effects have been linked to heart disease, ulcers, irritable bowel syndrome, Crohn's disease, mental illness (Kant et al., 1991), wound healing abilities (Dhabhar & McEwen, 1999), reactivation of latent herpes simplex virus, spread of mammary gland tumors (Padgett et al., 1998) atrophy of the thymus and enlargement of the adrenal glands (Seley, 1936, Basset & Cairncross, 1975). Stress has also been associated with the spread of cancer (Dhabhar & McEwen, 1999) and tumor growth (Kant et al., 1991). For example, breast cancer survival rates have been shown to increase dramatically in patients who receive group support or therapy and have better attitudes (fighting spirit) as compared to individuals who remain without support and have constant feelings of hopelessness (Watson el at., 1999). Researchers at Yale University found correlation between brain activity and stress levels by examining subjects suffering from coronary artery disease. Using positron emission tomography (PET scan) to show blood flow in the brain, researchers showed hyperactivation and deactivation of certain parts of the brain in patients with high stress levels or depression as compared to control patients suffering the same disease but coping with less stress (Soufer et al., 1998). Autonomic nervous system monitoring, a measurement of stress levels, has been shown to have a strikingly linear relationship to increasing viral load levels in HIV infected men after

undergoing highly active antiretroviral therapy (HAART immunotherapy) (Cole et al., 2001). Stress has also been linked to the reoccurrence of induced irritable bowel syndrome in mice. Qiu et al. found that stress facilitates the reactivation of colitis as compared to control (non-stressed) animals (Qiu et al., 1999). Wound healing is also affected by stress as found with a study of caregivers. Caregivers to family members with debilitating illnesses exhibited slower wound healing abilities when a punch biopsy wound (small circular incision) was placed on their arms. In contrast to controls (non-caregivers of approximately the same age, race, sex, health etc) caregivers took 24% longer to heal (Kiecolt-Glaser et al., 1995, Kiecolt-Glaser et al., 2003).

Stress perception causes activation of the HPAA, as previously stated in the introduction. The hypothalamus, the master gland, triggers the release of CORT and catecholamines, such as epinephrine, norepinephrine and dopamine. CORT controls the physical manifestations involved in the response (heart, lungs, immune system) while catecholamines control our emotional reaction to stress. Catecholamines affect the frontal sections of the brain, altering short term memory, rational thought and concentration (Hyman, 1998) in order to quickly react without thinking.

The immune system reacts to stress by directing the spleen to release white and red blood cells. Red blood cells will increase the oxygen capacity while white cells help the body handle the repercussions of the confrontation (Fox, 1993). Classically, antibody reactions are enhanced while cell-mediated immune functions are suppressed, as this has been the most common pattern seen with CORT secretion (Steinman, 2004). As stated previously, CORT appears to shift the T cell/ B cell response to enhance humoral immunity. The common cytokines associated with stimulating B cells are IL-4 and IL-10 and the common cytokines associated with triggering cell-mediated immunity are IFN_{γ} and IL-2. Phase 1 results, which modeled TH1 and TH2 cytokine secretion demonstrated the classic cytokine profiles for both types of TH cells. IL-2 is mainly associated with T cell growth and controlling T cell progression through the cell cycle. IFN_{γ} activates mononuclear phagocytes, neutrophils, natural killer cells and vascular endothelial cells, up-regulates the expression of MHC class I and II molecules and promotes T cell differentiation. IL-4 is a regulator of mast cells and IgE immune reactions and includes the stimulation of B cell proliferation and TH2 differentiation. IL-10 is an immunosuppressive cytokine which alters the ability of monocytes and macrophages to synthesize pro-inflammatory cytokines (IL-1, TNFα, IL-6), as well as IFN_{γ} (Gesser et al., 1997, Qin et al., 2001, Ding et al., 2000) and thus, indirectly reduces cytokine production by TH1 cells.

These studies provide additional evidence that stress leads to an alteration in immune function. Although these data were preliminary, they suggest a possible mechanism that may contribute to a disruption in the balance of the TH1/TH2 cell ratio. In both animal studies discussed here lymphocytes from the stressed animals appeared to have alterations in their cytokine profiles. One animal study (phase 2) suggested that lymphocytes from the stressed animals that were housed alone had less intracellular IL-4 when compared to stressed animals that were housed in pairs or control animals that were not stressed. These findings have several possible explanations. First, these lymphocytes may have less intracellular IL-4 because they are producing less of the cytokine (at the transcriptional or translational level). Second, they may have less of the cytokine intracellularly because more is being secreted into the environment. If more IL-4 (a TH2 cytokine) is secreted these findings suggest a shift towards a humoral (antibody production) or TH2 response, the pattern attributed to the effects of CORT. In order to address which scenario is more likely the extracellular concentrations of the TH cytokines were examined in the next study (phase 3). Although this study used a different species of animals the study also indicated that stress alters the production of the TH2 cytokines. However, in this study there was no difference in extracellular concentration of IL-4 produced by lymphocytes from either stress group (isolated or paired) when compared to

control animals, but the extracellular level of IL-10 was reduced in both groups of stressed animals when compared to controls. Since IL-10 inhibits macrophage function decreased levels of IL-10 may lead to an increase in macrophage activity. Since macrophages are key mediators of phagocytosis an increase in macrophage activity may lead to damage of healthy tissue during an inflammatory response, an important component of innate immunity. Increased macrophage proliferation may also offer an explanation for the enlarged spleens found in these animals. Additional studies need to be performed to address whether animals subjected to this stress paradigm exhibit an increase in the inflammatory response.

Although it was not the main focus of this immunologic study, we see evidence of social contact playing a role in mediating an animal's response to stress. In the first animal study (phase 2) lymphocytes from social animals (housed in pairs) subjected to stress exhibited similar IL-4 levels as the control animals, whereas in the second study (phase 3) lymphocytes from social animals subjected to stress exhibited IL-10 secretion patterns similar to the lymphocytes from the isolated (housed alone) stressed animals. Therefore, the addition of an additional stressor in the second animal study may have decreased the ability for the social stressed animals to compensate. Current theory behind sociality suggests that social contact, such as touching or stroking, stimulates the release

of oxytocin. Oxytocin, or the 'cuddle chemical' is a neuropeptide released by they hypothalamus. Classically oxytocin has been shown to stimulate lactation and uterine contractions in women (Uvnas-Moberg et al., 1998) and penile erection in men (Melis et al., 1986). Oxytocin is linked to sexual emotions and soothing feelings. It has been suggested that oxytocin also plays a significant role in socialization and bonding. Their studies demonstrate subjects with lowered blood pressure, decreased metabolic rate, increased glucose storage, reduced locomotor activity and increased pain threshold demonstrated increased oxytocin levels. Taken as a whole, this has lead to the theory that oxytocin may be the 'anti-stress drug', combating the fight or flight response (Uvnas-Moberg et al., 1998). The studies conducted at the University of Richmond were done in conjunction with Dr. Kelly Lambert at Randolph Macon College (RMC). Dr. Lambert conducted field tests prior to sacrificing the animals. Observations demonstrated that social stressed animals (inferred higher oxytocin levels due to socialization) were more active in field tests and more explorative compared to isolated stressed animals, and therefore suggest that social contact may alleviate some of the stress effects within these subjects.

In summary, these studies provide additional support that stress leads to a disruption in the balance of the ratio of TH1 and TH2 lymphocytes which may impact the immune response. No changes in the TH1 cytokines were detected

in these experiments as a direct response to stress (CUSP); however, changes in the TH2 cytokines (IL-4 and IL-10) were detected. An increase in IL-4 secretion from lymphocytes of stressed animals could lead to an increase in the humoral response at the expense of the cell-mediated response. The reduced levels of IL-10 that were secreted by lymphocytes from stressed animals may lead to an increase in macrophage activity in these animals. Increasing macrophage activity may leads to damaging inflammatory responses. These changes in the cytokine profiles have the potential to cause shifts in the T cell balance and impair a host's ability to respond to an infection or to maintain homeostasis.

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